THE COMPLEX INTERACTION BETWEEN PORCINE CIRCOVIRUS TYPE 2 AND THE PIG'S IMMUNE SYSTEM

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List of abbreviations

AEC	3-amino-9-ethylcarbazole
BAL	broncho-alveolar
BBTV	banana bunchy top virus
BCV	bovine circovirus
bp	base pair
CAV	chicken anemia virus
CD	colostrum deprived
CDCD	caesarean derived/colostrums deprived
cDNA	copy DNA
CFDV	coconut foliar decay virus
CoCv	columbid circovirus
ConA	concanavalin A
CysA	cyclosporin A
dpi	days post inoculation
D-MEM	Dulbecco's modified Eagle's medium
ED_{50}	50% effective dose
hpi	hours post inoculation
IF	immunofluorescence
Ig	immunoglobulin
FBS	foetal bovine serum
FCM	foetal cardiomyocytes
FITC	fluorescein isothiocyanate
IFN-α	interferon-alpha
IFN-γ	interferon-gamma
IL	interleukin
IP	intra-peritoneal
IPMA	immunoperoxidase monolayer assay
IM	intra-muscular
kDa	kilo Dalton
KLH	keyhole limpet hemocyanin
Mab	monoclonal antibody
MEM	minimal essential medium
mRNA	messenger RNA
Nab	neutralizing antibody
NLS	nuclear localization signal
ON	oronasal
ORF	open reading frame
PAM	porcine alveolar macrophage
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBS+	PBS supplemented with calcium and magnesium
PDNS	porcine dermatitis and nephropathy syndrome
PCR	polymerase chain reaction
PCV1	porcine circovirus type 1
PCV2	porcine circovirus type 2
PMWS	postweaning multisystemic wasting syndrome

PPV	porcine parvovirus
PRCV	porcine respiratory coronavirus
PRRSV	porcine reproductive and respiratory syndrome virus
RF	replication form
RPMI	rosswell park memorial institute
RT-PCR	reverse transcriptase PCR
SC	subcutaneous
SCSV	subterranean clover stunt virus
SD	standard deviation
SEM	standard error of the mean
SPF	specific pathogen free
SN	sero-neutralisation
SWC	swine work cluster
$TCID_{50}$	50% tissue culture infectious dose
TNF-α	tumour necrosis factor alpha
TTV	TT virus
UV	ultra-violet
VLP	virus like particle

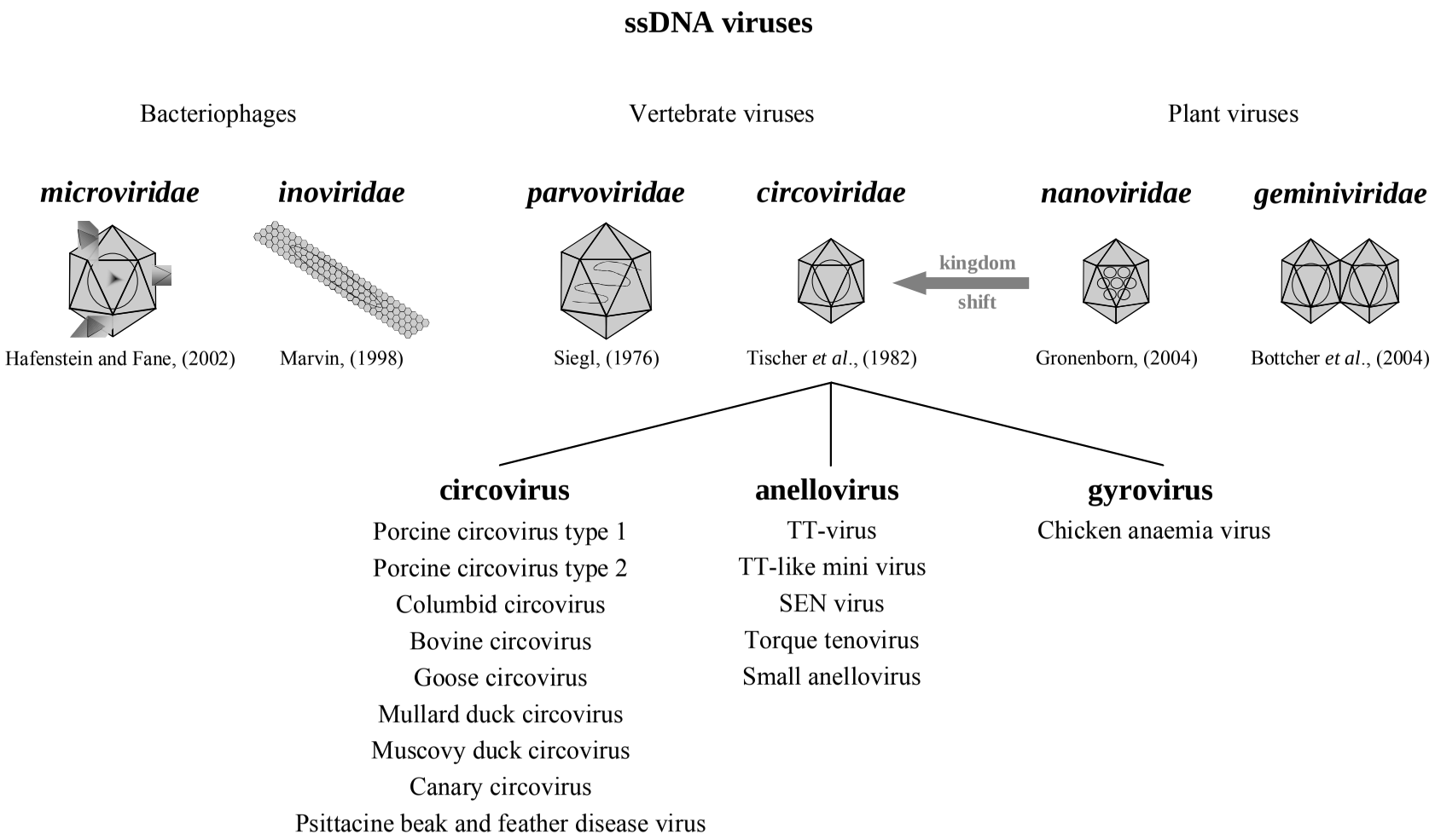
1. Introduction

1.1. CLASSIFICATION OF THE SSDNA VIRUSES

A broad range of viruses with a single stranded DNA (ssDNA) genome has been identified so far. These viruses have been found in vertebrates as well as in plants and bacteria. An overview of the ssDNA viruses is presented in Figure 1. Two families of ssDNA viruses have been found in vertebrates: the *parvoviridae* and the *circoviridae*. The *parvoviridae* are a long-known family of viruses that has members infecting many species. The family of *circoviridae* was more recently discovered and new members of this family are being identified until today. This family contains 3 different genuses: the circoviruses, the gyroviruses and the anelloviruses. The classification of new members of the circoviridae in these genuses is made based on their size and genomic organisation. Only one gyrovirus has been characterized, the chicken anaemia virus (CAV) isolated for the first time by Yuasa *et al*. (1979). Recently viruses with similar characteristics as CAV (Hino, 2002) have been isolated from humans and classified in the genus of the anelloviruses. The first isolation of a human anellovirus was made from a patient (initials T.T.) with post-transfusional hepatitis (Nishizawa *et al*., 1997) and was named TT-virus. Two more human anelloviruses were characterized in 2000: TT-like mini virus (Takahashi *et al*., 2000) and SEN virus (Primi *et al*., 2000).

The genus with the highest number of members is the genus of the circoviruses. Members of this genus were isolated from several avian and mammalian species. Porcine circovirus type 1 (PCV1) was the first circovirus to be identified (Tischer *et al*., 1982) followed by the psittacine beak and feather disease virus (Pass *et al*., 1984). In 1998, a second type of the circovirus was found in association with the postweaning multisystemic wasting syndrome, a new emerging disease in pigs at that time (Ellis *et al*., 1998). This virus was named porcine circovirus type 2 (PCV2). Many circoviruses have only recently been characterized such as the bovine circovirus (Nayar *et al*., 1999), goose circovirus (Soike *et al.*, 1999), Columbid circovirus (Woods *et al*., 1993) and duck circovirus (Soike *et al*., 2004).

Figure 1. Classification of the single stranded DNA (ssDNA) viruses



1.2. MORPHOLOGY AND GENOMIC ORGANISATION OF THE CIRCOVIRUSES

In 1982, Tischer *et al.* detected the first porcine circovirus (PCV1) as a contaminant of the porcine kidney cell line PK-15 (ATCC-CCL13). This new virus was very small in size (17 nm) and did not have an envelope. It was found to contain one single-stranded circular DNA molecule of approximately 1760 bases. In 1998, a new porcine circovirus was identified that showed 76% DNA homology with PCV1. This new virus was named PCV2 (Morozov *et al*., 1998). Morphology and genomic organisation were shown to be identical for both porcine circoviruses.

Analysis of the DNA molecule showed that it was ambisense and contained 11 possible open reading frames (ORF's). Until now, proteins from only two of these ORFs have actually been detected in infected cells. These two ORFs (ORF1 and ORF2) code for three different proteins. It is uncertain if these proteins are the only ones that are expressed by the virus since more viral mRNAs have been detected in PK-15 cells infected with the virus (Bratanich and Blanchetot, 2002; Cheung, 2003). ORF2 situated on the viral strand of the genome, codes for the capsid protein (Nawagitgul *et al*., 2000). The capsid of the virus is built only out of this protein (Crowther *et al*., 2003). When expressed in baculovirus systems, recombinant ORF2 proteins spontaneously form virion-like particles (VLPs) (Kim *et al*., 2002), indicating that the assembly of the virion capsid is very efficient. ORF 2 is the region in the genome that shows the highest variation between PCV1 and PCV2 with an amino acid homology of 65% (Morozov *et al*., 1998). The other characterized viral proteins are the non-structural Rep proteins. These proteins are associated with replication of the viral genome. The genetic information for these proteins is located in ORF1, situated on the complementary viral DNA strand. Through a frame shift, this single ORF is able to code for two different proteins, assigned Rep and Rep' (Mankertz *et al*., 1998; Mankertz *et al.*, 2001). Rep and Rep' proteins join and form a complex that is involved in replication of the viral genome (Steinfeldt *et al*., 2001). The DNA sequence of ORF1 is highly conserved between PCV1 and PCV2 (amino acid homology of 86%) (Morozov *et al*., 1998). This is an indication for the importance of the Rep protein complex for survival of the viruses. The morphology and genomic organisation of PCV2 is shown in Figure 2.

In PK-15 cells it was observed that viral antigens appeared in the infected cells starting from 18 hours post inoculation (hpi). The PCV2 capsid protein was found mainly in the nucleus of the infected cells indicating that assembly of the virus occurred at that site. Starting from 30 hpi, the production of progeny virus could be demonstrated (Cheung and Bolin, 2002).

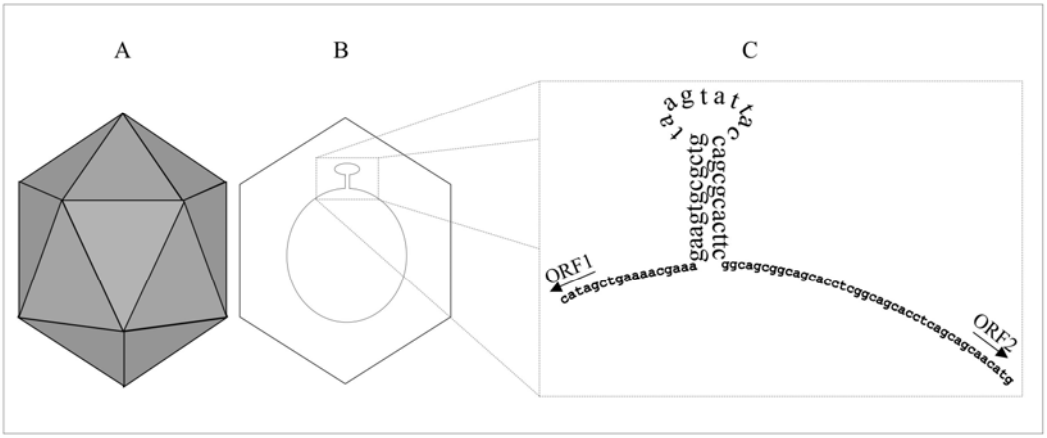


Figure 2. Morphology and genomic organisation of PCV2. A. The PCV2-virion consists of a capsid without envelope. B. The genome of PCV2 is a single stranded circular DNA molecule with a stem loop structure. C. A highly conserved nonanucleotide sequence is located on the top of the stem loop structure. Close to the stem loop structure the ORF2 starts on the viral strand coding for the capsid protein, in the other direction, on the complementary strand, starts ORF1 that codes for the Rep protein.

All members of the *circoviridae* share the property that they are not able to express a broad variety of viral proteins due to the limited genetic information they carry. Many of them are very wide spread in the population of their natural host (Sanchez *et al*., 2000; Rahaus *et al*., 2003; Ali *et al*., 2002; De Herdt *et al*., 2001) and many of these viruses are able to cause persistent infections (Bolin *et al*., 2001; Maggi *et al*., 2001; Imai *et al*., 1999). This indicates that disregarded their relatively simple structure, *circoviridae* are very successful in maintaining themselves in the population of their host.

1.3. REPLICATION CYCLE OF THE PORCINE CIRCOVIRUSES

The mechanism involved in attachment and internalization of porcine circoviruses into their host's cells is unknown. The cellular receptors that bind porcine circoviruses and by doing so initiate the replication cycle of the virus, remain to be discovered. Replication of the circovirus genome once it is inside the cell is believed to occur through a mechanism known as the rolling cycle replication (Meehan *et al*., 1997). The Rep/Rep' complex binds to the starting-point of the DNA-replication and initiates the DNA replication cycle. However, with the Rep/Rep' complex, no DNA-polymerase activity could be observed. The actual synthesis of new viral DNA is believed to be carried out by a cellular DNA-polymerase. This accounts for the fact that PCV1 requires replicating cells to complete its infectious cycle (Tischer *et al*., 1987). This rolling cycle replication has already been described in phages and plasmids (Novic, 1998) but also in plant viruses with a circular ssDNA genome: the *nanoviridae* (Groneborn, 2004) and *geminiviridae* (Laufs *et al*., 1995). Vertebrate circoviruses seem to have a high homology with these plant viruses. They have similar sizes, structures and genetic organisations and some sequences in their genomes are almost identical. One of these nearly identical sequences is the nonanucleotide sequence localized on the top of the stem loop structure. This highly conserved domain was proven to be the starting point of the rolling cycle replication (Meehan *et al.*, 1997; Niagro *et al.*, 1998). The nonanucleotide sequences of different vertebrate and plant viruses are presented in Table 1. The similarities between circoviruses that infect vertebrates and nanoviruses that infect plant are so striking that it is believed that the *circoviridae* are actually derived from these *nanoviridae*. Through a kingdom shift, a nanoviruse is thought to have infected a vertebrate host and to have recombined with a vertebrate virus (possibly a calicivirus) present in that host at that time. This way, a completely new virus was created. Luckily such a kingdom shift is a very rare event during evolution (Gibbs and Weiller, 1999).

Table 1. Alignment of the nonanucleotide sequence of different vertebrate and plant viruses.

	Virus	Nonanucleotide sequence	Reference
Vertebrate viruses	porcine circovirus type 1	TAGTATTAC	Cheung, 2003
	porcine circovirus type 2	AAGTATTAC	Meehan *et al.*, 1998
	bovine circovirus	AAGTATTAC	Hamel and Nayar 2004
	psittacine beak and feather disease virus	TAGTATTAC	Kloet and Kloet, 2004
	columbid circovirus	TAGTATTAC	Mankertz *et al.*, 2000
Plant viruses	banana bunchy top virus	TATTATTAC	Harding *et al.*, 1993
	coconut foliar decay virus	TAGTATTAC	Rohde *et al.*, 1995
	subterranean cloves stunt virus	TAGTATTAC	Boevink *et al.*, 1995
	geminivirus	TAATATTAC	Lazarowitz *et al.*, 1987

1.4. PATHOGENESIS OF A PORCINE CIRCOVIRUS TYPE 2 INFECTION

Experimental PCV2-infection of pigs has been achieved by various inoculation routes. The main natural infection route of PCV2 is generally believed to be the oronasal route. In the events following the primary infection, many questions remain to be answered. Until today, it is still unknown where the primary replication of PCV2 takes place. As a result it is unknown which physical barrier between the inner body and the outside environment is important in the prevention of PCV2-infection. Consequently it is also unclear what kind of local immune response would be able to interfere with (re-)infection of the host. Starting from 7 days post experimental inoculation (dpi), a mainly cell-associated viremia could be detected (Pensaert *et al.*, 2004) resulting in the spread of the virus to all organs in the body. A quite interesting characteristic of PCV2 is that at later stages of the infection, the virus can be isolated from a high variety of organs (Ellis *et al.*, 2000; Magar *et al.*, 2000; Sanchez *et al.*, 2001). This indicates that PCV2 is able to replicate in many different cell types or that it replicates in cells that are commonly present in different organs.

The identification of the target cell for PCV2-replication has been the subject of many studies. Based on morphological identification, PCV2-antigens were detected mainly in monocytes/macrophages but also to a lesser extent in hepatocytes, different types of epithelial cells, endothelial cells, lymphocytes, muscle cells, fibroblasts and neurons (Rosell *et al*., 1999; Kennedy *et al*., 2000; Stevenson *et al*., 2001). In 2003, Sanchez *et al*. performed a series of studies in porcine foetuses and newborn piglets. In these studies, porcine foetuses were inoculated *in utero* with PCV2 at different gestational ages (57, 75, 92 and 104 days of gestation). Following the same inoculation protocol, 1-day-old gnotobiotic piglets were inoculated with the same infectious dose of PCV2. The population of PCV2-positive cells was determined both in foetuses and in newborn pigs and compared. They observed considerable differences in PCV2-infected cells between pre- and postnatally inoculated animals. In foetuses, the main target cells were found to be cardiomyocytes and macrophages in the heart, the main target organ in PCV2-infected foetuses, and hepatocytes and macrophages in the liver. In postnatal piglets, hearts were almost negative for PCV2 and if present, antigens were only detected in macrophages. In lungs and lymphoid organs, the majority of positive cells were of the monocyte/macrophage lineage. In the postnatally inoculated piglets, two patterns in PCV2-positive cells were observed (Sanchez *et al*., 2004). In most of the piglets, only macrophages were shown to contain PCV2 proteins (both structural and non-structural PCV2-proteins were detected). Infectious PCV2-titres in the organs of these piglets were low. In a minority of piglets, high PCV2-titres were detected. In these piglets, besides infected macrophages, also a population of $CD4^+$ T-cells, $CD8^+$ T-cells and B-cells were infected. This study was the first study to emphasize the difference in degree of PCV2-replication and to correlate this with differences in the infected cell populations. This observation was of major importance for this thesis. Sanchez and co-workers did not discriminate dendritic cells (DCs) from other cells of the monocyte/macrophage lineage. The interaction of PCV2 with DCs is currently under investigation by another research group. It was observed that DCs are infected by PCV2 but no progeny virus could be detected (Vincent *et al*., 2004). The authors hypothesize that DCs would play an important role in the dissemination of PCV2 throughout the body. It is not clear whether PCV2 alters the immunological functions of the DCs it infects. Although many cell types have been claimed to contain PCV2 antigens *in vivo*, there is no known type of primary porcine cells that can be used to produce high titres of PCV2 *in vitro*.

Starting from 14 days after experimental inoculation, the number of PCV2 DNA copies decreased in most of the inoculated pigs. The start of the decrease in PCV2 replication coincided with the first detection of antibodies against PCV2 (Ladekjær-Mikkelsen *et al*., 2002). Other studies report the first detectable anti-PCV2 antibodies starting between 14 and 21 days post inoculation (dpi) (Progranichnyy *et al.*, 2000; Bolin *et al*., 2001; Harms *et al*., 2001). Only one study reports the existence of PCV2-neutralizing antibodies. These antibodies were detected starting from 28 dpi (Prognanichnyy *et al*., 2000). Since PCV2 is endemically present in most of the farms (Sanchez *et al*., 2000), most of the piglets under field conditions will receive maternally derived antibodies (MDA) against the virus. In the absence of infection, the MDA will decrease below the detection limit of standard serology assays between the age of 7 to 8 weeks (Labarque *et al*., 2000). Under field conditions the seroconversion is most typically observed at the age of 6 to 10 weeks in the presence of low titres of MDA (Labarque *et al*., 2000, Meerts *et al*., 2004). The onset and efficacy of the cellular immune response of the host towards PCV2-infection remain undocumented. The crucial role of the adaptive immunity in controlling the replication of the virus however, has clearly been demonstrated since artificial immunosuppression of gnotobiotic pigs by Cyclosporin A (CysA) treatment before and during PCV2-infection resulted in a dramatic increase in PCV2-titres in the pigs (Krakowka *et al*., 2002).

When literature is reviewed, indications are found that the immune response of the pig against PCV2 is not always capable of efficiently removing PCV2 from the body. In a number of studies, experimentally PCV2-inoculated piglets developed exceptionally high PCV2-titres compared to their group mates (Kennedy *et al*., 2000; Ladekjær-Mikkelsen *et al*., 2002; Sanchez *et al*., 2004). In two of these studies the level of PCV2 replication was followed in time by determining infectious titres in lymphoid organs of pigs euthanised at different time points (Sanchez *et al*., 2004) or by determining the number of PCV2 genome copies in the blood of pigs at different time points (Ladekjær-Mikkelsen *et al*., 2002). It was observed that PCV2-replication evolved with reasonable variation during the first 10 days of infection. Starting from approximately 14 dpi two different PCV2-replication patterns appeared in both of these studies. One group of pigs (3/4 and 6/10) showed a decrease of the level of PCV2 replication after 14 dpi. A second group of pigs (1/4 and 4/10) suffered a significantly higher PCV2-replication. PCV2-replication in these pigs even intensified after 14 dpi. In one study (Ladekjær-Mikkelsen *et al*., 2002) these pigs with high PCV2 replication

died of PCV2-associated disease (postweaning multisystemic wasting syndrome) before the end of the study. This observation indicates that some pigs are less able to remove the virus or virus-infected cells from their body. The most credible explanation for this event would be a difference in the efficiency of the immune response to cope with the replicating virus, rendering a limited fraction of pigs more susceptible to PCV2-replication and subsequently to the development of PCV2-associated disease. Revealing the mechanism responsible for this difference in susceptibility, would be an important step in understanding the pathogenesis of a PCV2-infection.

1.5. PORCINE CIRCOVIRUS TYPE 2 RELATED SYNDROMES

1.5.1. Postweaning Multisystemic Wasting Syndrome

Clinical presentation

After the first description of the porcine circovirus now known as porcine circovirus type 1 (PCV1) (Tischer *et al*. 1982), the virus was demonstrated to be endemically present in the pig population. At that time, PCV1 was not associated with any disease in pigs. Experimental PCV1-inoculations in seronegative pigs indicated that PCV1 only caused a subclinical infection (Tischer *et al*., 1986; Allan *et al*., 1995). These findings resulted in a lack of interest and need for further investigation of the virus. This vision was dramatically reconsidered when in 1998 a new type of porcine circovirus was identified (Morozov *et al*. 1998, Ellis *et al*., 1998). The new virus was assigned PCV2 and was found in association with a new emerging disease in pigs: the postweaning multisystemic wasting syndrome (PMWS), described for the first time by Harding (1996). High titres of PCV2 were isolated from a broad range of tissues of clinically affected animals (Allan and Ellis, 2000). The clinical presentation of this syndrome was characterized by severe growth retardation or weight loss in pigs after weaning. Figure 2 shows a group of PMWS-affected pigs. Besides the obvious signs of wasting, the most frequently observed pathological lesions in affected pigs were pneumonia and enlargement of lymph nodes. In a lesser proportion of cases also the kidneys or the liver were affected (Segalés and Domingo, 2002).

Figure 2. Example of PMWS-affected pigs

The pathological lesions described above are not typical for PMWS only but can be found in various chronical diseases. Therefore the observed lesions were further histologically characterized. Histopathological examinations revealed an interstitial inflammation in many affected organs (lungs, kidney, liver and colon) (Segalés and Domingo, 2004). A somewhat more pathognomonic microscopical lesion was found in the lymphoid tissues. As well in spleen as in lymph nodes, a severe depletion of lymphocytes was observed combined with an infiltration of monocytes into the lymphocyte areas. Furthermore, multinucleate giant cells could be detected in these tissues (Morozov *et al*., 1998; Rosell *et al*., 1999). The depleted cell populations in lymphoid organs of PCV2-infected pigs were characterized as B-cells and $CD4^+$ or $CD8^+$ T-cells (Shibahara *et al*., 2000; Sanchez *et al*., 2004). B- and T-lymphocytes were depleted not only from lymphoid tissues, in PMWS-affected pigs also a decrease in circulating B- and T-lymphocytes was observed (Darwich *et al.*, 2002; Nielsen *et al*., 2003). These changes in lymphocyte populations have been interpreted as indications for the immunosuppressive capacities of PCV2 (Rosell *et al*., 1999; Darwich *et al*., 2004; Segales *et al*., 2004). However, no mechanism of

immunosuppresion due to a PCV2-infection has been described until now, neither has any functional test revealed an influence of PCV2 on the general immunological capacity of its host. The depletion of lymphocytes has also been observed in association with circovirus-infections in other species such as pigeons and finches (Woods *et al*., 1994; Shivaprasad *et al*., 2004). In Chicken anemia virus (CAV) a suppressive effect of the virus on the immunity of the host has been revealed. In CAV-infected animals, it has been shown that the virus disabled the host to generate pathogen-specific T-cells (Markowski-Grimsrud and Schat, 2003).

Diagnosis

In order to differentiate PMWS from other chronical diseases causing a similar clinical picture in weaned pigs, Sorden (2000) proposed a way to diagnose PMWS based on the presence of clinical, pathological and histopathological lesions, combined with the demonstration of PCV2 in the affected organs. Using this definition, a positive diagnosis of PMWS could be made on an animal level. The presence of a single PMWS-affected pig in a herd however, does not embody the severity of the problem in that herd. In independent studies, single PMWS-affected pigs could be diagnosed in healthy farms with normal mortality rates, that did not experience important economic losses due to PMWS (Rodriguez-Arrioja *et al*., 2003; Meerts *et al*., 2004; Segalés personal communication). Therefore, a PMWS-affected farm was theoretically defined by Segalés *et al.* (2003) as a farm with an increased prevalence of PMWS-affected pigs compared to the historical background. Since the prevalence of PMWS is never being followed on a healthy conventional farm with the proper diagnostic tools, this definition might be more useful for scientific purposes than for everyday use by swine practitioners. This is the reason why scientists are still looking for a better definition of a PMWS-affected farm.

Prevalence and economical importance

The importance of PMWS rapidly increased in most Western-European countries during the late nineties. In 2000, the first diagnosis of PMWS was made in Belgium (Vyt *et al*., 2000). Contrastingly, PCV2 was shown to be present in the pig population long before PMWS was diagnosed. Archived sera originating from pigs from 1967

were tested and found to contain antibodies against the virus (Sanchez *et al*., 2000). This indicates that the association between PMWS and PCV2 has to be addressed with caution. Therefore, a study was performed to monitor PCV2-infections in PMWS-affected and healthy farms in Belgium and to detect correlations with the appearance of the disease (Meerts *et al*., 2004).

In this study, the prevalence of PMWS affected pigs was determined in three PMWS affected farms and in four control farms with no suspicion of PMWS in Belgium. The diagnosis of PMWS was based on the combined presence of four criteria: the presence of growth retardation, gross lesions and histopathological lesions suggestive for PMWS and the presence of high PCV2 titres in lymphoid organs. In PMWS affected farms, 2% of the piglets died of PMWS during the observation period. In the control farms, one out of 1000 piglets was found to be affected with PMWS. Serological examinations showed that all piglets became infected with PCV2 on all farms and no differences in serological profiles were seen between farms or litters with or without PMWS-affected piglets. This study showed that at that time PMWS was clearly not a problem of major importance in the Belgian pig population while at the same time the disease was causing high economical losses in other Western European countries. It could be concluded that the prevalence of PMWS in a population was influenced by unknown regional differences in the pig industry and not by the prevalence of PCV2. Another important conclusion in this study was the fact that a single PMWS-affected pig could be found in a healthy farm. This indicated that PMWS can be present in a farm without causing a lot of damage and that the experience of a PMWS-problem in a farm, might be inflicted by an increase in the number of PMWS-affected pigs, rather than by an introduction of the disease. The low prevalence of PMWS in Belgium described in this study was quite contrasting with the reports of high economic losses due to the disease in other European countries such as France and the United Kingdom. At the time of this description, two other European countries, Denmark and Sweden, were also reporting a low importance of PMWS in their pig herds. However, in both countries a dramatic increase in PMWS-affected farms was observed between 2003 and 2004 (Hassing *et al*., 2003; Wallgren *et al*., 2004).

From this study, 2 important questions rise: 1. what causes the difference between Belgium and its surrounding countries with regard to the prevalence of PMWS in their pig herd and 2. what mechanism is important in inducing PMWS in some pigs, while their littermates, although they are raised in identical conditions, remain completely unaffected. In this thesis some answers will be given to solve a part of this puzzle.

1.5.2. Porcine circovirus type 2 associated abortion

Field observations

In 1999, PCV2 was for the first time isolated from a litter of aborted foetuses (West *et al*., 1999). In one foetus, a diffuse myocarditis was associated with an extensive immunohistochemical staining for PCV2 antigens. From these aborted foetuses, no other agents that are being associated with reproduction problems in sows or abortions, could be isolated. After this first description of PCV2-induced abortion, several other descriptions were made. A common factor in many of these cases was the fact that the affected herd experienced problems at the time when they housed a high number of sows that were possibly seronegative for PCV2. In one case, a Specific Pathogen Free (SPF) farm known to be free of the virus got infected with PCV2 (Ladekjær-Mikkelsen *et al*., 2001), resulting in abortion of several sows. In two other occasions, new startup farms with new populations of gilts got affected by PCV2-associated abortion (O'Connnor *et al*., 2001; Farnham *et al*., 2003). From these data, it could be concluded that PCV2-related abortion was most likely to occur in PCV2-seronegative sows that got infected with PCV2. Regarding the endemic spread of PCV2 in nearly all pig farms (Sanchez *et al*., 2000), this may be considered as a rare event.

Experimental reproduction

In a series of experimental transuterine inoculations, Sanchez *et al*. (2001) investigated the susceptibility of foetuses at different gestational ages to PCV2-infection. The PCV2-titres obtained from inoculated foetuses were negatively correlated with the gestational age at the time of inoculation. Only in foetuses

inoculated at 57 days of gestation, gross lesions were observed (congestion of internal organs). These lesions were not observed in foetuses inoculated at later stages of gestation (75 or 92 days). This study clearly showed that porcine foetuses are susceptible for PCV2 and possibly die of the consequences of the infection.

In an attempt to demonstrate vertical transmission of the virus towards the foetuses, pregnant PCV2-seronegative gilts were inoculated with PCV2 (Pensaert *et al*., 2004). In this study, spread of the virus towards the foetuses could not be demonstrated. Recently however, another study described experimental reproduction of abortion by inoculation of PCV2 seropositive sows with PCV2 (Nielsen *et al*., 2004). In this study, 1 out of 2 inoculated pregnant sows farrowed at 118 days of gestation. The litter contained one dead-dorn and several unthrifty piglets. Pre-colostral antibodies against PCV2 were detected in sera of these piglets and PCV2 antigens could be retrieved from their organs. These studies indicate that vertical transmission of PCV2 to the foetuses after infection or re-infection of the sow is possible but does not occur in every pregnant sow that is (re-)infected with PCV2. Field surveys indicate that PCV2-associated reproductive failure does occur under field conditions but represent only a small fraction of the total number of reproductive disorders diagnosed in pig farms (Bogdan *et al.*, 2001; Kim *et al*., 2004).

However, the importance of PCV2 in inducing reproductive disorders could be underestimated. It was recently demonstrated that early stages of porcine embryo's become susceptible to PCV2-infection as soon as they are hatched out of the zona pellucida (Mateusen *et al*., 2004). From this study, it could be hypothesized that infectious PCV2, transmitted through semen during mating or artificial insemination, would be able to infect these early stages of embryonic development and result in early embryonic death. PCV2 genomic material has already been detected in semen of boars (Hamel *et al.*, 2000; Larochelle *et al*., 2000). Further research will have to point out if this association of PCV2 with early embryonic death is purely academic, or if it does occur in field conditions.

1.5.3. Others

PMWS and PCV2-associated abortion share the property that they can be reproduced, albeit with varying success, by inoculating pigs in specific conditions with PCV2. Besides these diseases, others have been mentioned in association with a PCV2 infection in pigs. However none of these syndromes have been experimentally reproduced, rendering the link between the disease and the virus controversial. A list of all syndromes and clinical signs that have been associated with PCV2 infection in the past, is presented in Table 2

Table 2. Overview of the diseases that have been associated with PCV2 infections.

Disease	Reproduced by experimental PCV2-inoculation?	Reference
Postweaning multisystemic wasting syndrome	yes	Harding, 1996
Abortion	yes	West *et al.*, 2000
Porcine dermatitis and nephropathy syndrome	no	Rosell *et al.*, 2000
Congenital tremor	no	Stevenson *et al.*, 2001
Exudative epidermitis	no	Wattrang *et al.*, 2002
Porcine respiratory distress complex	no	Harms *et al.*, 2002
Proliferating and necrotizing pneumonia	no	Harding, 2004
Enteritis	no	Kim *et al.*, 2004

The best described syndrome in this list is a syndrome that was observed in pigs starting from the mid-nineties. Pigs, usually in the fattening unit, showed a systemic necrotizing vasculitis resulting in skin lesions on the caudoventral part of the body, in combination with glomerulonephritis (Hélie *et al.*, 1995; Thibault *et al.*, 1998). Based on the predominant lesions, the disease was named Porcine Dermatitis and

Nephropathy Syndrome (PDNS). PCV2 was detected in tissues of PDNS-affected pigs (Rosell *et al*., 2000). The etiologic involvement of PCV2 in this disease is currently under debate since it was observed that PCV2 viral loads in PDNS-affected animals were not higher compared to the load in subclinically infected animals (Olvera *et al*., 2004). From this observation it could be concluded that the lesions associated with PDNS are not very likely due to an acute PCV2-infection since in that case high PCV2 titres should be present. When the lesions observed in affected pigs are compared to lesions seen in other animal species, they seem to match with lesions typically seen in association with a type III hypersensitivity reaction (Janeway *et al*., 2001). An indication for the involvement of PCV2 in the disease was found in the fact that PDNS-affected pigs showed significantly higher antibody-titres against the virus compared to subclinically infected animals (Wellenberg *et al*., 2004). This finding supports the hypothesis of a type III hypersensitivity since high antibody-titres against PCV2 would be able to form complexes that could initiate the hypersensitivity reaction. In PDNS-affected farms, the morbidity and mortality rates due to the disease are quite low (Thomson *et al*., 2002). However, since PDNS often affects pigs at the end of the fattening period, the economical consequences of the disease can be considerable. The syndrome seems to gain in importance in the Western European countries (Elbers *et al*., 2000).

1.6. EXPERIMENTAL MODELS TO REPRODUCE THE POSTWEANING MULTISYSTEMIC WASTING SYNDROME

Starting from the first detection of PCV2 in PMWS-affected pigs, researchers have been continuously investigating the role of PCV2 in the pathogenesis of the disease. In order to fulfil Koch's postulates, the reproduction of the disease by inoculation of the virus in susceptible pigs was mandatory. The results of these experiments were in many occasions very discouraging. When PCV2-replication was monitored in experimentally inoculated pigs, the only constant finding was the very high variation in replication between pigs (Balash *et al*., 1999; Allan *et al*., 2000a; Allan *et al*., 2000b; Kennedy *et al*., 2000; Ladekjær-Mikkelsen *et al*., 2002; Sanchez *et al*., 2003; Sanchez *et al*., 2004). Also the reproduction of PMWS-associated gross lesions and/or clinical symptoms showed high variation. Only a limited number of studies described the

successful reproduction of PMWS-associated gross lesions and clinical symptoms by inoculating pigs with PCV2 only (Kennedy *et al*., 2000; Bolin *et al*., 2001; Ladekjær-Mikkelsen *et al*., 2002; Okuda *et al*., 2003; Allan *et al*., 2003). The number of studies describing PCV2-inoculations in pigs without reproducing PMWS are much more abundant (Allan *et al*., 2000a; Krakowka *et al*., 2000; Krakowka *et al*., 2001; Allan *et al*., 2000b; Rovira *et al*., 2002; Krakowka *et al*., 2002; Sanchez *et al*., 2003; Opriessnig *et al*., 2003; Opriessnig *et al*., 2004a; Resendes *et al*., 2004; Sanchez *et al*., 2004; Opriessnig *et al*., 2004b). In all of the above-mentioned studies, pigs with no detectable titres of MDA against PCV2 were used. Seroconversion of the inoculated pigs against PCV2 was always detected, indicating that the variation in successfully reproducing PMWS by inoculating pigs with PCV2 was not due to a difference in susceptibility of the pigs to PCV2-infection. When the degree of PCV2-replication is compared between pigs from different studies by means of virus titres in affected organs, it is observed that certain high virus titres in one study were associated with disease, whereas in another study, equally high titres were found in clinically healthy animals. This indicates that the reproduction of the disease was not only associated with the level of virus replication, but also with unidentified variables in-between different experiments. One lesion was constantly observed in all studies. The depletion of lymphocytes and infiltration of monocytes in lymphoid organs in the presence or absence of clinical symptoms or macroscopical lesions, were always correlated with the presence of high PCV2 titres in these organs. The conclusion that could be drawn from this was that the microscopical lesions observed in PMWS-affected pigs could be induced by PCV2-infection but that for the reproduction of the macroscopical lesions and clinical signs associated with PMWS, other factors had to be considered.

In a series of experimental studies, the influence of secondary infections on the reproduction of PMWS in combination with a PCV2 infection was determined. Many of these secondary infections were selected based on their high prevalence in piglets at the time they experience PCV2 infections and PMWS under field conditions. However, the first successful identification of a possible cofactor was obtained by coincidence. In 1999, Allan *et al*. inoculated gnotobiotic piglets with a PCV2-stock that was accidentally contaminated with porcine parvovirus (PPV). In this experiment it was observed that pigs inoculated with both viruses had a higher chance of developing PMWS compared to pigs that were inoculated with a PCV2-stock without PPV. To check whether this was specific to the combination of PCV2 and PPV, another dual

inoculation was performed. In this second study, PCV2 was inoculated alone or in combination with the porcine reproductive and respiratory syndrome virus (PRRSV). Here again a higher chance of developing PMWS was observed in the dually inoculated pigs compared to the control pigs that were only inoculated with PCV2 (Allan *et al*., 2000b; Rovira *et al*., 2002). Pigs that were inoculated with both viruses showed a higher replication of PCV2.

At the time when these dual inoculations were performed, it was unknown which mechanism, which was activated both by PPV and PRRSV infection, was responsible for the enhanced PCV2-replication. To check if this could be accredited to the induction of an immune response, gnotobiotic pigs were injected with keyhole limpet hemocyanin (KLH) in Freund's incomplete adjuvant at the same time of PCV2-inoculation (Krakowka *et al*., 2001). By doing so, a reproducible immune response was induced in these pigs. All gnotobiotic pigs that received KLH together with the PCV2-inoculation developed PMWS, while the control pigs that were only inoculated with PCV2 only experienced a subclinical infection. This was the first study to indicate that an immune response of the host was able to influence the intensity of PCV2-replication and the induction of clinical symptoms upon this infection. Which part of the immunity was responsible for this enhancement, was still unkown. Surprisingly, the effects induced by KLH-injection in gnotobiotic piglets could not be reproduced in conventional specific pathogen free (SPF) pigs when the same protocol was applied (Ladekjær-Mikkelsen *et al*., 2002). In this experiment, PMWS was observed in both the KLH-inoculated and the mock-inoculated animals. This difference in susceptibility to develop PMWS upon PCV2-infection between gnotobiotic and conventional pigs might be caused by their different background.

A number of other studies were performed in which PCV2-inoculated pigs were inoculated or treated with other factors. A third infectious agent that was found to exacerbate the clinical outcome of a PCV2 infection in experimentally inoculated pigs was *Mycopasma hyopneumoniae* (Opriessnig *et al.*, 2004). This infection might be of importance in the pathogenesis of PMWS in field conditions since *Mycoplasma hyopneumoniae* infections can occur in very young piglets. Injection of experimentally PCV2-inoculated pigs with vaccine-adjuvant (Resendes *et al*., 2004) or a bacterin (Opriessnig *et al*., 2003) did not result in the induction of clinical signs but they did induced a higher PCV2 replication. An overview of the studies in which the influence of possible cofactors was investigated, is given in Table 3.

Table 3. Overview of studies describing the clinical outcome of PCV2-inoculation in pigs with or without secondary infections or treatments.

Study reference	Status of pigs	Secondary inoculation or treatment	Wasting observed in …	
			PCV2-inoculated pigs	PCV2-inoculated pigs + additional inoculation/treatment
Kennedy *et al.*, 2000	conventional	porcine parvovirus	yes	yes
Allan *et al.*, 2000	CD	porcine parvovirus	no	yes
Krakowka *et al.*, 2000	CDCD	porcine parvovirus	no	yes
Krakowka *et al.*, 2001	CDCD	keyhole limpet hemocyanin	no	yes
Allan *et al.*, 2000	CD	PRRSV	no	yes
Rovira *et al.*, 2002	conventional	PRRSV	no	yes
Ladekjær-Mikkelsen *et al.*, 2002	conventional	keyhole limpet hemocyanin	yes	yes
Krakowka *et al.*, 2002	CDCD	cyclosporin A	no	no
Opriessnig *et al.*, 2003	conventional	bacterin	no	no
Opriessnig *et al.*, 2004	conventional	porcine parvovirus	no	yes
Resendes *et al.*, 2004	conventional	Vaccine adjuvant	no	no
Opriessnig *et al.*, 2004	conventional	*M. Hyopneumoniae*	no	yes

Although it is now generally accepted that PCV2-infection is required for the induction of PMWS, it is also accepted that secondary factors play a crucial role. The mechanism by which these cofactors influence the clinical outcome of a PCV2-infection remains to be elucidated. This thesis will focus on this question.

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2. Aims of the thesis

Porcine circovirus type 2 (PCV2) is a recently discovered virus in pigs that is endemically present in the population. Infections of pigs with this virus, have been associated with various diseases and syndromes. Two of these syndromes have been successfully reproduced by inoculating susceptible animals with PCV2, the postweaning multisystemic wasting syndrome (PMWS) and PCV2-associated abortion. The target cells of PCV2 replication in postweaning pigs and foetuses have already been identified but the replication cycle of the virus in these cells remains to be established.

Postweaning multisystemic wasting syndrome has been associated with a continuously high level of PCV2 replication in pigs typically between 4 and 14 weeks of age. Upon experimental inoculation of seronegative pigs, only a minority of pigs experienced this high and persisting PCV2 replication. The mechanism that determines the difference in degree of PCV2 replication between pigs is unknown. This thesis aims to identify host-specific factors that are able to influence the replication of PCV2 *in vivo* during the early stages of the infection, but also factors that lie at the base of the persistent infection that is seen in naturally PMWS-affected animals as well as in experimentally inoculated pigs.

The aims of this thesis can be more specified as follows:

- To determine the replication cycle of PCV2 in its natural target cells and in fully susceptible porcine cell lines, in order to investigate the influence of cytokines on PCV2-replication *in vitro* and subsequently *in vivo*.
- To characterize the immunological response of the host against a PCV2-infection in order to find correlations with the evolution of PCV2-replication in that host.
- To confirm observations, made in this thesis during experimental PCV2-inoculations, in naturally PMWS-affected animals.

3.

Replication of porcine circovirus type 2 in alveolar macrophages and cardiomyocytes

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Summary

In this *in vitro* study, the replication kinetics of porcine circovirus type 2 (PCV2) in porcine alveolar macrophages (PAM) and fetal cardiomyocytes (FCM), two target cells *in vivo*, was compared with that in PK-15 cells. Cultures were inoculated with either the postweaning multisystemic wasting syndrome (PMWS)-associated strain Stoon-1010 or the abortion-associated strain 1121. Viral proteins were visualized and virus production was determined. In PK-15 cells, the capsid protein was expressed between 6 and 12 hours post inoculation (hpi), it relocated to the nucleus between 12 and 24 hpi. At that time Rep protein was also detected in the nucleus. This sequence of events also occurred in FCM and PAM but nuclear localized antigens appeared later (48 hpi) and in a lower percentage of cells. In PAM, clear differences in susceptibility were seen between pigs. In PAM from two out of five tested pigs, no nuclear localized antigens could be detected, whereas in PAM from three other pigs they were seen in up to 20% of the antigen-positive cells. Virus production was observed in PK-15 but not in PAM or FCM cultures. In a second study, the replication kinetics of seven different PCV2 strains were compared in PK-15 cells. It was shown that 2 abortion-associated strains had different replication kinetics in comparison with PMWS or porcine dermatitis and nephropathy syndrome associated strains. With the abortion-associated strains, a higher number of infected cells was observed after 24 hours, the fraction of these cells with nuclear localized antigens was lower compared to the other strains.

Introduction

The genus circovirus in the family *Circoviridae* contains 2 porcine viruses: porcine circovirus type 1 (PCV1) and porcine circovirus type 2 (PCV2). PCV1 was first described by Tischer *et al*. (1982) as a contaminant in the porcine kidney cell line PK-15. Experimental inoculations with this virus in susceptible pigs did not result in clinical signs or pathological lesions (Tischer *et al*., 1987; Allan *et al*., 1995) and as a result, PCV1 was generally recognised to be non-pathogenic. PCV2 was first isolated in 1997 from a piglet affected by the postweaning multisystemic wasting syndrome (PMWS) (Ellis *et al*., 1998). More recently, this virus was also detected in aborted and stillborn foetuses (West *et al*., 1999; Ladekjær-Mikkelsen *et al*., 2001). Experimental inoculations with PCV2 strain Stoon-1010 that was isolated from a case of PMWS (Ellis *et al*., 1998) and with PCV2 strain 1121, isolated from aborted foetuses (Meehan *et al*., 2001) have confirmed the crucial role of PCV2 in the reproduction of both syndromes (Allan and Ellis 2000; Sanchez *et al*., 2001).

The circovirus structure is characterized by its relative simplicity. The 1.8 kb single-stranded genome consists of a single circular ambisense DNA molecule (Tischer *et al*., 1982). Transcripts of 2 open reading frames have been characterized in PCV1-infected cells. Open reading frame 1 (ORF1), situated on the viral DNA strand, is transcribed into two collinear transcripts: Rep and Rep' (Mankertz *et al*., 1998; Steinfeldt *et al*., 2001). Both proteins form a complex that is involved in the replication of the virion ssDNA replication (Mankertz and Hillebrand, 2001). Open reading frame 2 (ORF2), situated on the complementary DNA strand, codes for the capsid protein (Nawagitgul *et al*., 2000) that has a molecular weight of 30-35 kDa (Cheung and Bolin, 2002; Nawagitgul *et al*., 2000). In order to express the capsid protein, it is believed that the complementary strand has to be synthesized through the intermediate double-stranded replication form (RF) as described for geminiviruses (Gutierrez 1999; Tischer and Bukh, 1988). Since circoviruses do not code for their own DNA-polymerase for the production of the double-stranded RF, they depend on a host DNA-polymerase to complete the replication of their genome. The requirement for host DNA-polymerases accounts for the observation that PCV1 requires PK-15 cells in the S-phase of the mitosis cycle to complete its infectious cycle (Tischer *et al*., 1987).

Immunophenotyping of the target cell of PCV2-replication *in vivo* has shown that the susceptible cell population in the pig depends on the stage of development of the host at the time of infection. In *in utero* inoculated fetuses, infected cells were identified as cardiomyocytes, hepatocytes and macrophages during early gestation and mainly macrophages towards the end of gestation (Sanchez *et al*., 2003). Postnatally, in the majority of pigs showing low or moderate PCV2-replication, the infected cells were demonstrated to be macrophages ($SWC3^+$/$sialoadhesin^+$). In contrast in the low percentage of pigs showing high PCV2 replication, infiltrating monocytes ($SWC3^+$/$sialoadhesin^-$) were also positive for PCV2 antigens (Sanchez *et al*., 2004).

In the continuous cell line PK-15, a productive infection has been demonstrated, starting with detection of viral antigens in the cytoplasm and the nucleus of infected cells at 18 hours post inoculation (hpi) and a release of progeny virus at 32 hpi (Cheung and Bolin, 2002). This replication cycle differs with *in vitro* observations in PCV2-inoculated porcine monocytes and macrophages in which PCV2 capsid protein was detected in the cytoplasm but for which no evidence of further virus replication was found (Allan *et al*., 1994; Gilpin *et al.*, 2003). In the absence of any evidence of active virus replication, it was presumed that the presence of capsid protein inside the cell was due to accumulation of viral antigens derived from the inoculum rather than from expression upon transcription of the virus' genome.

Although the genetic similarity between PCV2 strains from cases of PMWS or abortion is high (>95%), differences in biological properties between strains may still exist. Small genetic differences may have important consequences in tropism of viruses as has been described for closely related viruses such as transmissible gastroenteritis virus and porcine respiratory coronavirus (Rasschaert *et al*., 1990). Consequently, although a spectrum of closely related PCV2 genotypes have been characterized from a wide range of clinical syndromes, little information is available regarding possible differences in pathogenicity or tropism.

The aims of this study were (i) to examine the PCV2 replication kinetics in porcine fetal cardiomyocytes and porcine alveolar macrophages, two major target cells *in vivo* and to compare it with replication kinetics in PK-15 cells and (ii) to investigate if differences in replication kinetics exist between PCV2 strains.

Material and methods

Viruses and cells

Seven PCV2 strains were enclosed in this study. The origin and passage history of these strains are shown in Table 1.

PCV negative PK-15 cells were seeded on glass coverslips (BELLCO®, Vineland USA) at 150,000 cells per 3.15 cm^2 in minimal essential medium (MEM) with Earle's salts (GIBCO BRL®, Grand Island, USA) supplemented with 5% fetal bovine serum (FBS), 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. Cells were maintained at 37°C in the presence of 5% CO_2 for 24 hours to obtain a 50% confluent monolayer.

Fetal cardiomyocytes (FCM) were obtained by trypsinisation of heart muscle tissue of foetuses at 75 to 100 days of gestation. Fetal hearts were collected and washed in PBS. The heart muscle tissue was minced and incubated for 5 minutes in PBS containing trypsin (Sigma, Bornem, Belgium) (2.5 mg/ml) at 37°C. Trypsinized cells were collected, filtered, cooled to 4°C in FBS and centrifuged at 300 x g for 10 minutes. The cell pellet was resuspended in MEM with Hank's salts (GIBCO BRL®, Grand Island, USA) with 10% FBS, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. The cells were then seeded onto coverslips and incubated at 37°C. After 24 hours of incubation, medium was replaced by MEM with 10% FBS, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. Cardiomyocytes were incubated at 37°C in the presence of 5% CO_2 until the culture reached 50% confluency. Cardiomyocytes were identified by immunofluorescence stainings with monoclonal antibodies directed against desmin (DAKO Diagnostics, Glostrup, Denmark). Cell cultures from fetal heart tissue contained 90-100% desmin-positive cells showing mitosis in culture.

Porcine alveolar macrophages (PAM) were isolated from lungs of 4-week-old conventional piglets as previously described (Labarque *et al*., 2000). Briefly, the lungs were flushed with cold phosphate-buffered saline (PBS). The washing fluid was centrifuged and cells were resuspended in cooled RPMI 1640 medium (GIBCO BRL®, Grand Island, USA) supplemented with 10% dimethyl sulfoxide (DMSO), 30% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin, frozen and stored in liquid nitrogen. For subsequent experimentation, PAM were thawed and seeded at

$1x10^6$ cells per glass coverslip. After 2 hours of incubation in RPMI 1640 medium supplemented with 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 1% non-essential amino acids 100x (GIBCO BRL®, Grand Island, USA) and 1 mM sodium pyruvate at 37°C in the presence of 5% CO_2, coverslips were washed to remove non-adherent cells and 1.5 ml of the above mentioned medium supplemented with 10% FBS was added. Batches of PAM derived from five different piglets were used in this study. Cells in lung washing fluids were found to contain > 95% macrophages, using the monoclonal antibodies SWC3 and 41D3 directed against cells of the monocyte/macrophage lineage and against differentiated macrophages respectively (Blecha *et al*., 1994; Vanderheijden *et al*., 2003).

Table 1. Origin and passage history of porcine circovirus 2 strains enclosed in this study. PMWS: postweaning multisystemic wasting syndrome; PDNS: porcine dermatitis and nephropathy syndrome

Strain	**Origin**		**Passage level in PK-15 cells**
	isolated from …	**country (reference)**	
Stoon-1010	PMWS affected piglet	Canada (Ellis *et al*., 1998)	25
1121	aborted fetuses	Canada (Meehan *et al*., 2001)	21
1103	aborted fetuses	Canada (Meehan *et al*., 2001)	20
48285	PMWS affected piglet	France (Meehan *et al*., 2001)	16
VC2002	PMWS affected piglet	Belgium (Meerts *et al*., 2004)	16
1206	PMWS affected piglet	Belgium	23
1147	PDNS affected piglet	UK (Meehan *et al*., 2001)	15

Inoculation

Cultures of PK-15 cells, FCM or PAM were inoculated with $10^{4.2}$ $TCID_{50}$ of the respective strains, suspended in 0.5 ml MEM (PK-15, FCM) or RPMI (PAM). After 1 hour of incubation at 37°C in the presence of 5% CO_2, cell cultures were washed once with medium and further incubated in fresh medium. A mock-inoculated control was included in each experiment.

Fixation and stainings

At 0, 6, 12, 24, 36, 48 and 72 hours post inoculation (hpi) (cell cultures inoculated with reference strains 1010 and 1121) or 0, 12, 24, 48 and 72 hpi (cell cultures inoculated with other strains), the supernatant of inoculated cultures was collected, centrifuged at 15,000 x g for 10 minutes and stored at –70°C until virus titration. Cell cultures on coverslips were fixed in acetone at –20°C for 20 minutes, dried and stored at –20°C.

PCV2 antigen-positive cells were stained by subsequent incubation with biotinylated, purified monospecific porcine polyclonal antibodies against PCV2 and streptavidin-FITC (Molecular Probes, Leiden, The Netherlands). The total number of PCV2 antigen-positive cells was determined by counting the positive cells present in a total of 50,000 to 100,000 cells, depending on the confluency of the culture. The positive cells were further sub-divided into two fractions: cells with exclusive cytoplasmic localization of antigens and cells with nuclear and cytoplasmic localization of antigens. All experiments were repeated three times and mean values were calculated. To confirm that a positive signal was due to expression of viral proteins and not due to viral antigens taken up by the cell, PK-15 cells and PAM were inoculated with UV-treated strain Stoon-1010 and stained as described above.

Western blot analysis of the porcine polyclonal antibodies showed that the antibodies detected a 35 kDa protein which corresponds to the molecular size of the PCV2 capsid protein. No specific bands could be detected in the region of the Rep or Rep' protein, indicating that the polyclonal antibodies only recognized the capsid protein. This result was confirmed by performing double immunofluorescence stainings using the biotinylated porcine polyclonal antibodies and mouse monoclonal antibodies (Mab) directed against PCV2 proteins (F210 directed against Rep protein and F217 or F190 against capsid protein) (McNeilly *et al.*, 2001) on PCV2-infected

PK-15 cells. Mab F217 positive signals always colocalized with polyclonal antibody positive signals, indicating that both antibodies recognised the same protein. In contrast, some cells positive for F210 were not stained by the polyclonal antibodies, indicating that the polyclonal antibodies did not recognise the Rep protein. These findings correspond with the results in a previous study in which it was shown that porcine hyperimmune serum only reacted with capsid protein (Cheung and Bolin, 2002).

Expression kinetics of both known PCV2 proteins (capsid and Rep protein) were assessed using Mab F210 (directed against Rep protein) and F217 or F190 (against capsid protein). Bound Mab were visualised with goat-anti-mouse-FITC or goat-anti-mouse-Texas Red (Molecular Probes, Leiden, The Netherlands). Double immunofluorescence stainings were performed to detect both viral proteins and their colocalization in PK-15 cells. Inoculated cell cultures were incubated with Mab F210 (IgG_1) against Rep protein and F190 (IgG_{2b}) against capsid protein. Bound Mab were visualised with isotype specific secondary antibodies (Serotec, Oxford, United Kingdom). Hoechst 33342 (Molecular Probes, Oregon, USA) at a concentration of 10 μg/ml was used in all immunofluorescence stainings to visualize the nucleus. Viral antigen positive cells were counted by fluorescence microscopy. Digital images were made using a Leica TCS SP2 laser scanning spectral confocal system (Leica Microsystems GmbH, Heidelberg, Germany).

Virus titration of culture supernatant

Progeny virus titres in culture supernatants were determined by inoculation of 10-fold dilution series on PK-15 cells. After 72 hours of incubation at 37°C in the presence of 5% CO_2, supernatant was removed, cells were fixed in 10% paraformaldehyde in PBS and viral antigens were detected using an immunoperoxidase monolayer assay (IPMA), as described elsewhere (Meerts *et al*., 2004).

Results

PCV2 replication kinetics of reference strains Stoon-1010 and 1121 in PK-15 cells

Considerable differences were observed between replication kinetics of strains Stoon-1010 and 1121 (Figure 1). After inoculation with strain Stoon-1010, cells with viral antigens in the cytoplasm were detected starting from 12 hpi using the polyclonal antibodies and the numbers of positive cells increased in time. At 24 hpi, 0.3% of the cells expressed viral antigens, a maximum of 1.8% viral antigen positive cells was reached at 48 hpi. Starting from 24 hpi, nuclear localized viral antigens were seen in up to 25% of the positive cells. Inoculation with strain 1121 resulted in viral antigen-positive cells detectable from 6 hpi and rapidly increasing in time to 1.8% at 24 hpi and a maximum of 3.0% at 48 hpi. Starting from 12 hpi, nuclear localized viral antigens were seen in 1-5% of the positive cells. Foci of infected neighbouring cells were seen starting from 36 hpi with both strains. Inoculation with UV-inactivated PCV2 resulted in the absence of a signal after staining with polyclonal or monoclonal antibodies. The changes of antigen expression patterns in Stoon-1010 inoculated PK-15 are shown in Figure 2.

Figure 1. Number of porcine circovirus 2 antigen-positive PK-15 cells and antigen expression pattern at different time points after inoculation with strains Stoon-1010, 1121, 48285, 1103, 1206, 1147 and VC2002.

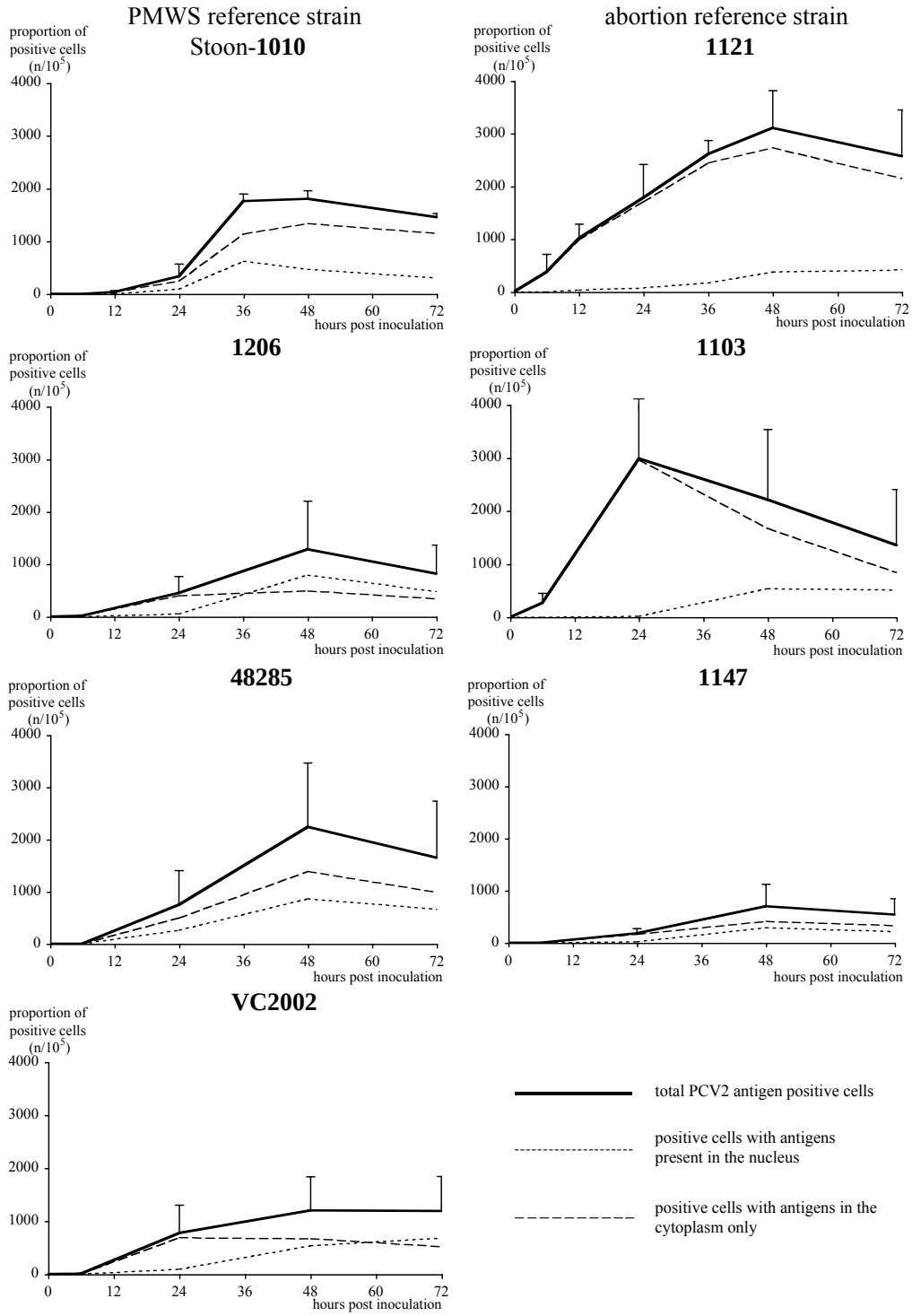
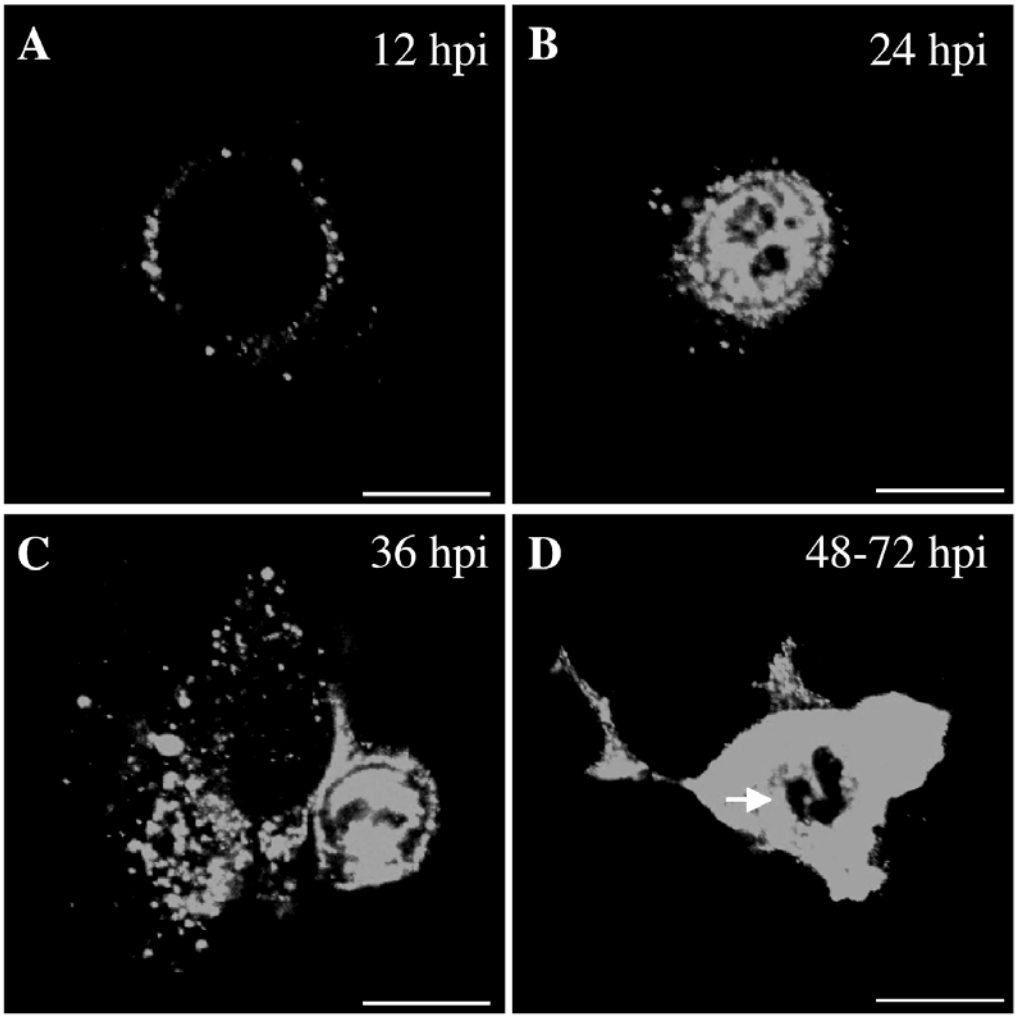


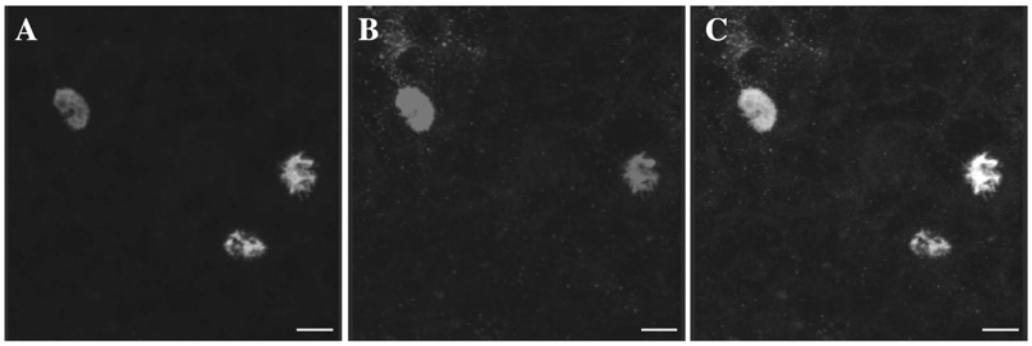
Figure 2. Changes in antigen expression patterns in porcine circovirus 2 infected PK-15 cells. Evolution in antigen expression pattern in Stoon-1010 inoculated PK-15 cell cultures, stained with polyclonal anti-PCV2 antibodies. A: PCV2 antigens in the perinuclear region of the cytoplasm at the early stage of infection (12 hpi). B: expression of PCV2 antigens in cytoplasm and nucleus (24 hpi). C: foci of infected cells (36 hpi). D: irregularly shaped cell with intense viral antigen positive cytoplasm and a viral antigen negative and deformed nucleus (arrow) (48 – 72 hpi). Bar = 30 μm



Mab F217 and F210 were used to detect respectively PCV2 capsid and Rep proteins in Stoon-1010 and 1121 inoculated PK-15 cells. Capsid protein was the first viral protein detected. In early stages, the protein was only seen in the perinuclear region of the cytoplasm. Starting from 12 hpi in 1121-inoculated and 24 hpi in Stoon-1010 inoculated cells, the capsid protein was also detected in the nucleus of infected cells. Starting from 48 hpi, irregularly shaped cells with condensed nuclei were seen.

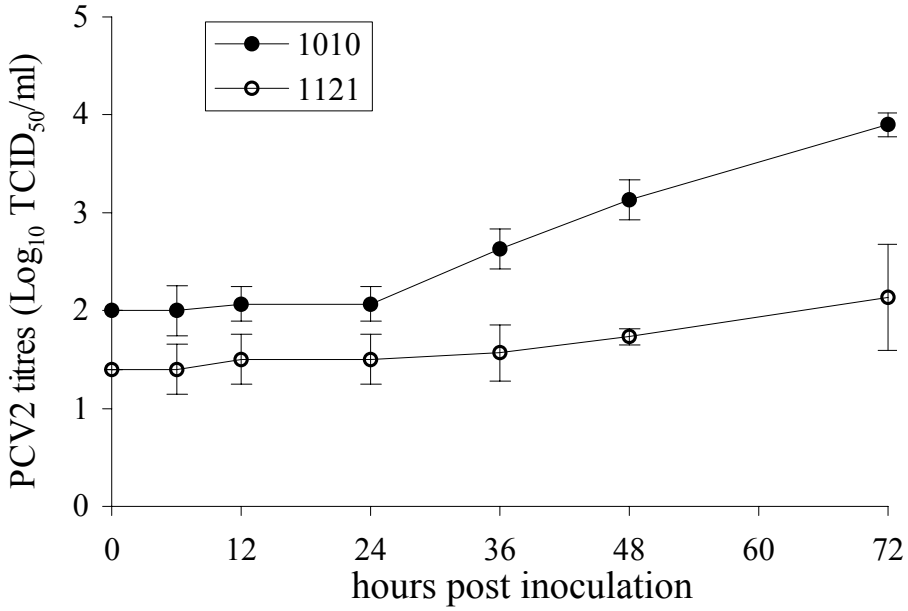
In these cells, the nucleus was negative for capsid protein while an intense signal was observed in the cytoplasm. During the early stages of cytoplasmic PCV2 capsid protein expression, Rep protein was not detected in infected cells. At 12 hpi in 1121 inoculated cultures and at 24 hpi in Stoon-1010 inoculated cultures, Rep protein was clearly apparent in the nucleus of infected cells. Rep protein was never detected in cytoplasm of infected cells except in low amounts in irregularly shaped cells starting from 48 hpi. Double IF stainings with monoclonal antibodies F210 and F190 showed that all cells with nuclear localized capsid protein, also contained Rep protein in their nucleus. In contrast a low fraction of cells (< 5% of cells with nuclear localized PCV2 antigens) was found to contain only Rep protein in their nucleus (Figure 3).

Figure 3. Expression of Rep and capsid protein in porcine circovirus 2 infected PK-15 cells at 72 hpi. A: PCV2 Rep protein is detected in the nucleus of 3 infected PK-15 cells. B: PCV2 capsid protein is detected in the nucleus of 2 infected PK-15 cells and in the cytoplasm of 2 other infected cells. C: merged image of both stainings showing 2 cells with both capsid and Rep proteins in the nucleus and one cell with only Rep protein in the nucleus. Bar = 30 μm



Virus titres in culture supernatants at different stages of infection are shown in Figure 4. A time-dependent rise of titres was seen starting from 36 hpi for both PCV2 strains, with a maximum titre of 2.1 $\log_{10}$ $TCID_{50}$ for strain 1121 and 3.9 $\log_{10}$ $TCID_{50}$ for strain Stoon-1010.

Figure 4. Evolution in porcine circovirus 2 titres in supernatant of PK-15 cell cultures inoculated with strains Stoon-1010 or 1121. Mean values and standard deviations are shown.

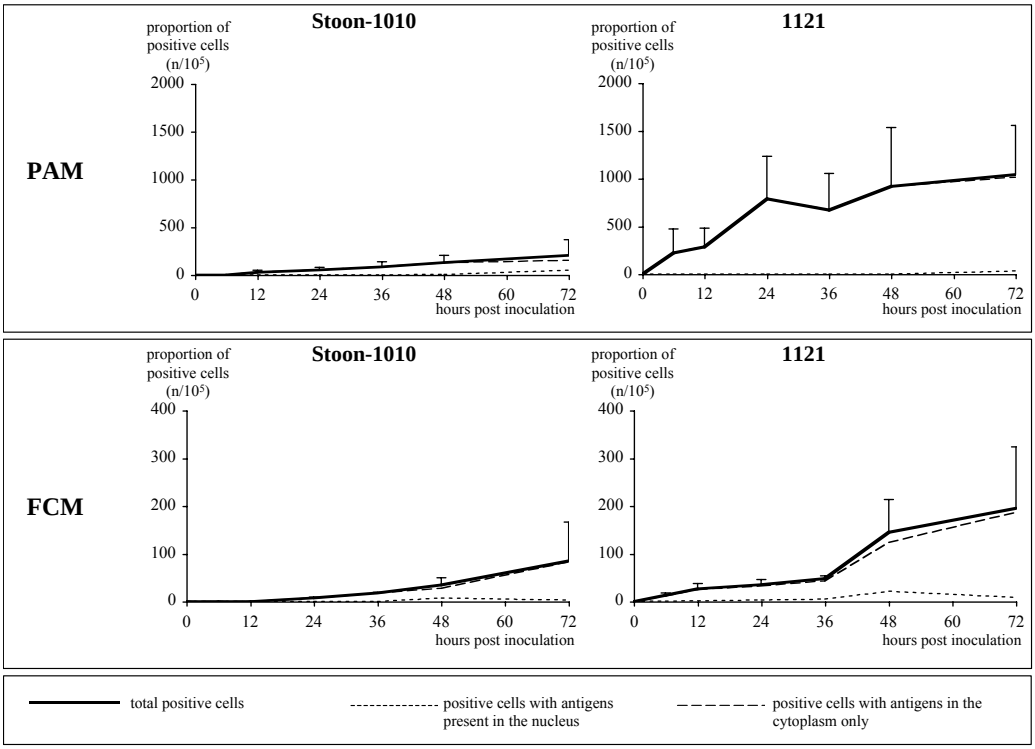


Evolution of the number of FCM and PAM expressing viral antigens upon PCV2 inoculation

The replication patterns of strains Stoon-1010 and 1121 in PAM and FCM are shown in Figure 5.

In FCM, a similar pattern was seen as in PK-15 cells although the percentage of viral antigen positive cells was approximately 10 times lower. In Stoon-1010 inoculated cell cultures, positive cells were detected starting from 24 hpi resulting in a limited number of cells expressing viral antigens in the nucleus at 48 hpi. Cell cultures inoculated with strain 1121 showed cytoplasmic positive cells starting from 6 hpi and nuclear positive cells starting from 36 hpi. For both strains, the number of positive cells was still increasing with both strains at 72 hpi.

Figure 5. Evolution of the number of porcine alveolar macrophages and fetal cardiomyocytes expressing porcine circovirus 2 antigens after inoculation with strains Stoon-1010 or 1121. The results in PAM represented in this figure, are the results obtained in one batch of PAM from one pig. In four other batches of PAM from other pigs, a lower fraction or no PCV2 antigen positive cells contained antigens in their nucleus.

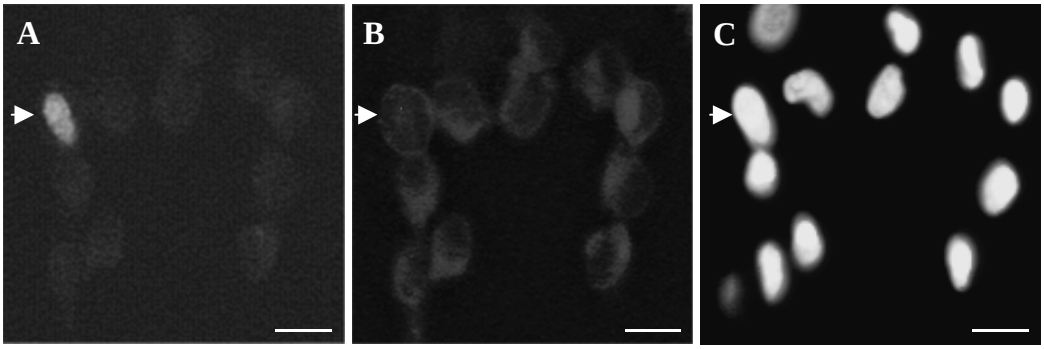


In PAM of one piglet, the number of viral antigen-positive cells increased in a time-dependent manner from 0.03% at 12 hpi to 0.25% at 72 hpi with strain Stoon-1010 and from 0.22% at 6 hpi to 1.1% at 72 hpi with strain 1121. Up to 48 hpi, antigens were exclusively localized in the cytoplasm and the signal increased in intensity with time. The first nuclear localization of viral antigens in PAM of this piglet was observed at 48 hpi (Stoon-1010) or at 72 hpi (1121). At 72 hpi, in Stoon-1010 inoculated cultures, 0.05% of the cells showed nuclear localized antigens (20% of the total number of positive cells) and in 1121 inoculated cultures, 0.03% of the cells showed nuclear localized antigens (3% of the total number of positive cells). The results obtained in this batch of PAM are shown in figure 5. Nuclear PCV2 antigen positive cells were confirmed to be macrophages by double immunofluorescence stainings with monoclonal antibodies F210 for the PCV2 Rep protein and SWC3 and

41D3 as shown in Figure 6. Of particular significance the number of PAM showing nuclear localization of PCV2 antigens varied strongly between batches of PAM derived from different piglets. In two batches of PAM, cells exclusively contained PCV2 antigens in their cytoplasm and nuclear localized PCV2 antigens were never seen. In two other batches, nuclear antigens were rarely seen (<1%) in the antigen positive cells, while in one batch up to 20% of the total PCV2 positive PAM contained antigens in the nucleus. Inoculation with UV-inactivated PCV2 resulted in the absence of a signal after staining with the polyclonal or monoclonal antibodies.

Supernatants of FCM and PAM cultures were titrated but no significant rise in virus titre was detected.

Figure 6. Porcine circovirus 2 infection in alveolar macrophages. Triple immunofluorescence staining showing nuclear localization of PCV2 Rep protein in a $SWC3^+$ macrophage. A: PCV2 Rep protein, B: SWC3, C: Hoechst visualizing the nucleus of the cell. Nuclear localization of PCV2 antigens remained a rare event since the majority of antigen positive PAM only contained antigens in the cytoplasm. Bar = 30 μm



Comparison of replication kinetics of different PCV2 strains in PK-15 cells

The replication kinetics of five additional strains are shown in Figure 1. Four strains (1206, 48285, VC2002, 1147) showed an evolution in number of viral antigen positive cells and in intracellular distribution of antigens similar to strain Stoon-1010, using the polyclonal antibodies. Some variation in the absolute numbers of infected cells was observed. Strain 1103, which is an abortion-strain from Canada, similar to strain 1121, showed similar replication kinetics as strain 1121. A rapid increase in

cytoplasmic positive cells was observed during the first 24 hours of the experiment. The percentage of infected cells showing nuclear localization of viral antigens in 1103 infected cell cultures was comparable with 1121 (1% at 24 hpi) infected cell cultures and lower compared to cell cultures inoculated with PMWS or PDNS-associated PCV2 strains (≥ 10% at 24 hpi).

Discussion

In the present study, new insights were gained in the replication characteristics of PCV2 in PK-15 cells and the natural target cells, alveolar macrophages and fetal cardiomyocytes. Furthermore, indications for biological differences between PCV2 strains were found in the replication kinetics of the strains in PK-15 cells.

PCV2 replication in PK-15 cells was studied as a reference and positive control for the full replication cycle of the virus. The earliest detectable viral protein in infected PK-15 cells was the capsid protein, which was mainly found in the perinuclear region of infected cells during the earliest stages of infection. Absence of immunofluorescence signal after inoculation with UV-inactivated PCV2 demonstrated that the early-detected capsid protein was newly produced in the infected cell, rather than derived from captation of viral antigens from the inoculum. ORF2, which codes for the capsid protein, is located on the complementary strand of the virus' genome (Nawagitgul *et al*., 2000) and can thus only be transcribed from the double stranded replication form. The early expression of capsid protein in PK-15 cells suggests that the complementary strand was formed very shortly after infection of the cell, as has been described for monopartite geminiviruses (Donson *et al*., 1984). Starting from 12 hpi, capsid protein was found in the nucleus of infected cells. At that time point also Rep protein was found in the nucleus of the infected cells. Although Rep protein, like any other protein, has to be produced in the cytoplasm, it was immediately observed in the nucleus of the infected cell, indicative for a strong affinity for the nucleus. At present however, no nuclear localization signal (NLS) has been described for this protein. Double immunofluorescence stainings for both PCV2 capsid and Rep protein showed that most of the cells with nuclear localized PCV2 antigens contained both capsid and Rep. A low percentage of cells contained only Rep

protein without expression of capsid (nuclear or cytoplasmic). This indicated that, although the capsid protein is the first viral protein detected in most of the infected cells, Rep protein can occasionally be the first expressed viral protein. The cellular or viral mechanisms that form the basis for this event remain to be elucidated. The fact that capsid protein was never found in the nucleus of an infected cell in the absence of Rep protein, although it can be abundantly present in the cytoplasm of that cell, suggests that the capsid interacts in some way with the Rep protein to cross the nuclear membrane. The inability of the capsid protein to reach the nucleus on itself in our study is surprising since Liu *et al*. (2001) identified a nuclear localization signal and detected the capsid protein in the nucleus upon transfection.

The rise in virus titre in culture supernatant at 36 hpi coincided with the appearance of the first foci of infected cells. This indicates that the full replication cycle of PCV2 in the PK-15 cell has a duration between 24 and 36 hours. These data agree with a previous study in which the first detectable progeny virus was found at 32 hpi (Cheung and Bolin, 2002). Starting from 48 hpi and increasing in numbers at 72 hpi, detaching cells were observed with capsid protein abundantly expressed in their cytoplasm. Only in these cells, Rep protein was detected in the cytoplasm, most likely as a result of leakage from the degrading nucleus. These cells showed irregular formed nuclei with condensation of genomic material, indicating that infection of PK-15 cells with PCV2 probably leads to cell death.

Similar viral protein expression patterns were observed in primary porcine cells and in PK-15 cells. In cardiomyocytes, PCV2 capsid protein and subsequently Rep protein were detected. The low absolute number of infected cells may be the reason for the absence of progeny virus in culture supernatant. Mitotic activity of cardiomyocytes *in vitro* is lower compared to PK-15 cells and this may explain why only 0.2 % of the cells were infected. This accurately reflects the situation *in vivo* where it was observed that with further development of the fetus towards parturition, the cardiomyocytes lose their susceptibility for PCV2 infection together with their ability to divide (Sanchez *et al*., 2003).

PCV2 capsid antigens have already been described in the cytoplasm of monocytes and alveolar macrophages (Gilpin *et al*., 2003). It was, however, questioned if this positive signal was due to transcription of the viral genome or due to accumulation of virus present in the inoculum. In the present study, capsid protein was detected in the cytoplasm of a low percentage of macrophages (<1%). The

number of positive cells increased in a time dependent manner. UV-inactivation of the inoculum resulted in absence of an immunofluorescence signal at any time after inoculation, indicating that the observed signal was due to expression of newly formed viral antigens. Limited numbers of infected macrophages ($SWC3^+/41D3^+$) contained PCV2 antigens in their nucleus. The low number of macrophages showing this nuclear staining is comparable with the *in vivo* situation, where antigens are mainly found in the cytoplasm of cells of the monocyte/macrophage lineage in PCV2 inoculated-piglets (Sanchez *et al*., 2004). Macrophages with nuclear expression of PCV2 antigens were not described in previous studies (Gilpin *et al*., 2003), which can be explained by the differences in origin of the cells or in the inoculation dose or duration. In the present study, nuclear expression of viral proteins was observed in PAM originating from some specific piglets only. It is unclear if this *in vitro* difference between piglets can form the base of a different clinical outcome of a PCV2 infection *in vivo*.

Replication of PCV2 in macrophages, which are fully differentiated cells, seems contradictory with the fact that the virus depends on the mitosis cycle of PK-15 cells and FCM to replicate in these cell types. The cell cycle dependency of PCV2 to complete its replication cycle is credited to the expression of DNA-polymerase expressed during the S-phase of the cell cycle (Tischer *et al*., 1987). Although macrophages do not divide, it has been shown that they can express high DNA polymerase activity in response to damage to their DNA (Williams *et al.*, 2002), an event which might be misused by PCV2 to complete its replication in these cells.

In this study, biological differences in-between PCV2 strains besides genetical differences were described for the first time. It is not fully understood if the contrasting behaviour of strains 1121 and 1103 in comparison with the other strains can be explained by their origin (abortion cases) or by the fact that the geographical regions where they were isolated, were close to each other. The importance of differences in the *in vivo* replication kinetics is not fully understood and will be the subject of further investigations.

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4. Influence of interferon-gamma on porcine circovirus type 2 infection

4.1. ENHANCEMENT OF PORCINE CIRCOVIRUS TYPE 2 REPLICATION IN PORCINE CELL LINES BY INTERFERON-GAMMA PRE AND POST-TREATMENT AND INTERFERON-ALPHA POST-TREATMENT

4.2. INCREASED PORCINE CIRCOVIRUS TYPE 2 REPLICATION IN GNOTOBIOTIC PIGS TREATED WITH CONCANAVALIN A

4.1.

Enhancement of porcine circovirus type 2 replication in porcine cell lines by interferon-gamma pre and post-treatment and interferon-alpha post-treatment

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Summary

Stimulation of the porcine immune system results in increased replication of porcine circovirus type 2 (PCV2) *in vivo*. In the present study it was investigated if a range of cytokines (IL-1, IL-6, IL-10, TNF-alpha, IFN-alpha and IFN-gamma) were able to influence PCV2-infection *in vitro*. No changes were observed in IL-1, IL-6, TNF-alpha or IL-10-treated cells. However, it was demonstrated that IFN-alpha and IFN-gamma influenced PCV2 infection in porcine kidney cells (PK-15) and porcine monocytic cells (3D4/31). IFN-gamma, added to the culture medium before, during or after inoculation, increased the number of PCV2-antigen positive cells with 418, 171 or 691% in PK-15 cells and with 706, 114 or 423% in 3D4/31 cells. IFN-alpha pre-treatment decreased the number of infected PK-15 cells. When it was added after inoculation, it enhanced PCV2 infection with 529% in PK-15 cells and 308% in 3D4/31 cells. The effect of both interferons on PCV2 infection was dose-dependent and could be blocked with IFN-alpha or IFN-gamma neutralizing antibodies. Leukocyte-derived porcine IFN-gamma induced a similar effect on PCV2-infection. Treatment of PK-15 cultures with IFN-gamma resulted in a 20 times higher production of progeny virus. Confocal microscopy studies showed that the enhancing effect of IFN-gamma on PCV2-infection was achieved by increased internalization of PCV2 virion-like particles (VLPs). Binding of the VLPs to the cell or expression kinetics of PCV2-proteins in infected cells, were not altered by IFN-gamma treatment. To our knowledge, this study reports the first enhancement of a viral infection by treatment with type I or type II interferons.

Introduction

Porcine circovirus 2 (PCV2) is a member of the *Circoviridae*. It is a very small virus with a relatively simple structure. The circular ambisense 1.7 Kb genome codes for two major proteins (Morozov *et al*., 1998). Open reading frame (ORF) 2 codes for the capsid protein (Nawagitgul *et al*., 2000) and ORF1 codes for two non-structural proteins Rep and Rep' which form a complex that is involved in replication of the viral genome (Mankertz and Hillebrand, 2001). Besides these proteins, no other viral proteins have been characterized yet. PCV2 does not code for its own DNA-polymerase and therefore it depends on cellular enzymes to complete its infectious cycle (Tischer *et al*., 1987). With its limited number of viral proteins, PCV2 is not well equipped to modify the intracellular conditions in favour of its own needs for replication, in contrast with many other more complex viruses (Izumi, 2004; Evers *et al*., 2004; Hay and Kannourakis, 2002). However, relatively simple viruses such as PCV2 might profit from specific conditions in the host that have been induced by other infectious agents. Recent observations indicate that PCV2 might do so. It has been shown that PCV2 was able to replicate better in a host when it was simultaneously inoculated with other viruses such as porcine reproductive and respiratory syndrome virus (PRRSV) (Allan *et al*., 2000a; Rovira *et al*., 2002) or porcine parvovirus (PPV) (Allan *et al*., 2000b; Krakowka *et al*., 2000; Opriessnig *et al*., 2004). These observations indicate that PCV2 profits from the reaction of the host to an infectious insult to increase its replication and in some studies to induce disease (Allan *et al*., 2000a; Rovira *et al*., 2002; Allan *et al*., 2000b; Krakowka *et al*., 2000; Opriessnig *et al*., 2004). PCV2-infection has primarily been associated with postweaning multisystemic wasting syndrome (PMWS), a condition in weaned pigs, characterized by severe growth retardation. The effect of a stimulation of the immune system on the replication of PCV2 and the induction of PMWS was investigated by Krakowka *et al*. (2001). They injected PCV2-inoculated gnotobiotic (caesarean-derived, colostrum-deprived) pigs with keyhole limpet hemocyanin (KLH) and by doing so, induced an immune response. This treatment was found to activate the replication of PCV2 and exacerbate the clinical outcome of the infection. Ladekjær-Mikkelsen *et al*. attempted to repeat this in conventional specific pathogen free pigs (Ladekjær-Mikkelsen *et al*., 2001) but were not successful. Pigs in this study that were not immune-stimulated also developed disease. When literature is reviewed, a

general observation is that gnotobiotic piglets are quite resistant to the induction of PCV2-associated disease when they are inoculated with PCV2 alone. Two studies describe the reproduction of PMWS in gnotobiotic pigs (Bolin *et al*., 2001; Okuda *et al*., 2003) whereas six studies failed to induce disease by inoculating PCV2 only (Krakowka *et al*., 2000; Krakowka *et al*., 2001; Krakowka *et al*., 2003; Sanchez *et al*., 2003; Sanchez *et al*., 2004; Meerts *et al*., 2005). It has already been shown that gnotobiotic pigs, compared to conventional pigs, have an impaired capacity to raise an immune response upon an infection due to the absence of regular stimulations of their immune system (Butler *et al*., 2002). It may be hypothesized that the higher resistance of gnotobiotic pigs to develop PMWS compared to conventional pigs, may be due to an impaired capacity to replicate PCV2 compared to conventional pigs caused by the altered immune response. The question arises if PCV2 is able to misuse an ongoing immune response to enhance its own replication in its host.

Comparative studies between germ-free and conventional mice have shown that germ-free animals react in a totally different way to infections with regard to the cytokine profiles they produce. They produce lower titres of pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) which are important in the earliest phases of the immune response. They produce significantly higher titres of interleukin-10 (IL-10), a cytokine to which mainly immunosuppressive properties are attributed (Souza *et al*., 2004; Duarte *et al*., 2004). Different cytokines are already known to influence the susceptibility of the host towards viral infections. The most studied and best-understood effect of cytokines on viral infections is the anti-viral effect of type I and type II interferons (Katze *et al*., 2002; Schroder *et al*., 2004). But also other cytokines such as TNF-α can influence the evolution of an infection (Calabrese *et al*., 2004). The effect of cytokines on PCV2-replication has never been investigated before *in vivo* nor *in vitro*.

The aim of the present study was to check the effect of a selected number of cytokines (IL-1, IL-6, TNF-α, IFN-α, IFN-γ and IL-10) on the replication of PCV2 *in vitro*.

Materials and methods

Cells, virus and inoculation

In a first set of experiments, PK-15 cells free of porcine circoviruses were used. The cells were seeded and maintained in culture medium containing 5% foetal bovine serum (FBS), 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin dissolved in minimal essential medium (MEM) (Gibco BRL®, Grand Island, USA). Cells were seeded at a concentration of 15 x 10^4 cells per ml of medium. For the detection of total number of viral antigen positive cells, 1.5 x 10^4 cells were seeded in every well of a 96-well microtiter plates (Nunc, Roskilde, Denmark). To detect the expression of specific viral antigens, 22.5 x 10^4 cells were seeded on sterile cover slips in Leighton tubes and finally progeny virus production assays were performed in 24-well plates (Nunc, Roskilde, Denmark) containing 15 x 10^4 cells per well.

In order to use cells more related to the target cells *in vivo*, the porcine monocytic cell line 3D4/31 (Weingartl *et al*., 2002) was used to confirm the effect of the different cytokines on the number of PCV2-infected cells. The 3D4/31 cell line was previously shown to be fully susceptible for PCV2-infection (Misinzo *et al*., 2005). These cells were maintained in a monocyte/macrophage medium containing a 1:1 mixture of RPMI-1640 (Gibco BRL®, Grand Island, USA) and Dulbecco's modified Eagle medium (DMEM) (Gibco BRL®, Grand Island, USA) supplemented with 10% foetal bovine serum, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin and 1% non-essential amino acids 100x (GIBCO BRL®, Grand Island, USA). These experiments were performed in 96-well plates containing 2 x 10^4 cells per well.

PK-15 and 3D4/31 cells were inoculated when they were 50% confluent (24 hours after seeding). All inoculations were performed with the same PCV2-strain (Stoon-1010) at a multiplicity of infection of 0.3. The virus was diluted in MEM for inoculation on PK-15 cells and in a 1:1 RPMI-1640/DMEM mixture for inoculation on 3D4/31 cells. Medium was removed from the cell cultures and cells were inoculated by incubation with virus for 1 hour at 37 °C in the presence of 5% CO_2. Afterwards, the inoculum was removed, cultures were washed twice with MEM or RPMI-1640/DMEM and new culture medium was added.

Due to the low number of PCV2 particles in virus stocks propagated in PK-15 cells, PCV2 VLPs were used to visualize attachment and entry of the virus into the infected cell. PCV2 VLPs, produced in Spodoptera frugiperda Q cells infected with baculoviruses containing PCV2 ORF2, were kindly provided by Dr. M. Bublot (Merial SAS, Lyon, France). 3D4/31 cells were seeded at 2.5 x 10^4 cells per well in monocyte/macrophage medium in an 8-well LAB-TEC® cover glass chamber slides (Nalge Nunc International, Naperville, USA) and incubated with VLPs as described earlier (Misinzo *et al*., 2005).

Recombinant porcine cytokines and neutralizing antibodies

Porcine recombinant IL-1α, IL-6, IL-10 and IFN-γ were purchased from R&D systems (Abingdon, UK). Porcine recombinant IFN-α was kindly provided by Dr. B. Charley (Institut National de la Recherche Agronomique, Department of molecular virology and immunology, France) (Lefevre *et al*., 1990). TNF-α was produced in L929 cells transfected with the pBMGNeo expression-vector containing the porcine TNF-α coding cDNA (Von Niederhausern *et al*., 1993). Activity of recombinant TNF-α was measured using the bio-assay described previously (Pauli, 1995). IL-1, IL-6, IL-10 and IFN-γ were dissolved according to the manufacturer's instructions in lipopolysaccharide free phosphate buffered saline (PBS) supplemented with 0.1% bovine serum albumin (Sigma, Bornem, Belgium) to a concentration of 10 μg/ml. Subsequently, the cytokines were diluted in MEM supplemented with 10% FBS to a concentration eight times higher than the highest final concentration used in the assays and stored at –70 °C.

Porcine IFN-α neutralizing mouse monoclonal antibodies (K9) were kindly provided by Dr. B. Charley. Porcine IFN-γ neutralizing mouse monoclonal antibodies (B12) (Kontsek *et al*., 1997) were kindly provided by Prof. Dr. B. Goddeeris (Laboratory of Physiology and Immunology of Domestic Animals, KULeuven, Belgium).

Leukocyte-derived porcine cytokines

To confirm the effects observed with recombinant porcine cytokines, leukocyte-derived porcine cytokines were included. Peripheral blood mononuclear cells

(PBMCs) were isolated from blood of a conventional 4-week-old pig by differential centrifugation on Ficoll Paque® (Amersham Bioscience AB, Upsalla, Sweden). These PBMCs were cultured in medium as described earlier and stimulated with 5 µg/ml concanavalin A (ConA) (Sigma, Heidelberg, Germany) (Verfaillie *et al*., 2001). Simultaneously, a culture of PBMCs was incubated in medium without ConA. After 16 hours of incubation at 37 °C in the presence of 5% CO_2, the supernatants were collected and centrifuged to remove cells and cell fragments. The concentration of IFN-γ in these supernatants was respectively 20.5 Units/ml (U/ml) (1U = 1 50% effective dose (ED_{50}) = 0.030 ng) and below the detection limit as determined by a porcine IFN-γ specific ELISA (Biosource, Nijvel, Belgium). Using a bioassay described by Rubinstein *et al.* (1981), IFN-α was not detected in these supernatants.

Effect of recombinant porcine cytokines on the total number of PCV2-antigen positive cells

The effect of the cytokines on the infection of PCV2 in PK-15 and 3D4/31 cells was determined by adding two-fold dilution series of the cytokines to the medium of the cells before, during or after the inoculation. Two-fold dilutions of all cytokines were used in this study. IL-1α (1U = 1 ED_{50} = 0.013 ng), IL-6 (1U = 1 ED_{50} = 2.5 ng), IL-10 (1U = 1 ED_{50} = 8 ng), TNF-α and IFN-α were used in concentrations ranging from 0.25 – 250 U/ml, IFN-γ was used in concentrations ranging from 0.25 to 1000 U/ml and IL-10 was used in concentrations from 0.13 to 125 U/ml. Cell cultures were either pre-treated with the cytokines for 24 hours before inoculation, treated during the inoculation (1 hour) or the cytokines were added in the medium after inoculation (until fixation). After 36 hours of incubation at 37 °C in an environment supplemented with 5% CO_2, after the first replication cycle of PCV2, the cells were dried and frozen at –20 °C. The plates were stained with an immunoperoxydase monolayer assay (IPMA) as described before (Meerts *et al*., 2005) and the number of PCV2-antigen positive cells was counted by light microscopy. In each plate, mock-treated cells (treated with MEM + 10% FBS without cytokines) were inoculated with an equal dose of PCV2. The average number of antigen positive cells in these wells was used as the reference and all results were expressed as a percentage of this reference.

Effect of IFN-γ on the attachment and internalization of recombinant PCV2-like virions

The attachment of PCV2 VLPs to untreated 3D4/31 cells and 3D4/31 cells pre-treated (24 hours) with 500 U/ml IFN-γ was studied as previously described by Misinzo *et al*. (2005). Briefly, treated and untreated 3D4/31 cells were chilled on ice and washed before PCV2 VLPs (2.7×10^{10} particles per 2.5×10^5 cells) were added and allowed to attach for 0, 1, 5, 10, 15, 30 and 60 minutes at 4 °C. Unbound PCV2 capsids were washed-off and cells were fixed with 3% (w/v) paraformaldehyde in phosphate-buffered saline with calcium and magnesium (PBS+) at room temperature for 10 minutes. In order to stain the PCV2 VLPs, cells were incubated with biotin-conjugated anti-PCV2 swine polyclonal antibodies followed by streptavidin-FITC (Molecular Probes, Leiden, The Netherlands) each for 1 hour at room temperature. Successive images from the apex to the base of a stained single cell were taken using a Leica TCS SP2 laser scanning spectral confocal system (Leica Microsystems GmbH, Heidelberg, Germany) linked to a Leica DM/IRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) and merged. The fluorescence area of individual fluorescence spots in images of stained PCV2 VLPs was calculated using image analysis software (SigmaScan Pro 5.0, Jandel Scientific GmbH, Erkrath, Germany) as described before. The fluorescence area of attached PCV2 VLPs per cell was counted for ten cells at each time point to establish their binding kinetics on 3D4/31 cells.

To study the influence of IFN-γ on internalization of PCV2 VLPs in 3D4/31 cells, PCV2 VLPs were incubated with 3D4/31 cells pre-treated for 24 hours with 500 U/ml IFN-γ or with non-treated 3D4/31 at 37°C. Thirty minutes after the addition of PCV2 VLPs on the cells, cells were fixed with a 3% solution of paraformaldehyde in PBS+ for 10 minutes. Thereafter, cells were washed with PBS+, and permeabilized with 0.1% Triton X-100 in PBS+. PCV2 VLPs were stained as described above. In order to visualize the cell border, an actin staining was performed by incubating the cells with Phalloidine-Texas red (Molecular probed, Oregon, USA) for 1 hour at 37°C. By confocal microscopy, the percentage of cells with PCV2 VLPs inside the actin rim was calculated and considered as the percentage of cells that were internalizing the VLPs. From these internalizing cells a digital image was taken through their centre and the percentage of fluorescing signal inside the actin rim was

calculated using image analysis software as described above. This was considered to be the percentage of internalized PCV2 VLPs in that cell.

Effect of IFN-γ on the expression kinetics of PCV2 proteins

To determine the effect of IFN-γ on the expression kinetics of PCV2 proteins (Capsid protein and Rep), PK-15 cells were inoculated on glass cover slips in Leighton tubes. After inoculation, the inoculum was removed, cultures were washed once in MEM and culture medium was added with or without 500 U/ml IFN-γ. At 0, 6, 12, 24, 36, 48 and 72 hpi the cells were fixed in methanol at –20°C. Afterwards, a triple immunofluorescence staining was performed to visualize both viral proteins and the cell nucleus as described previously (Meerts *et al*., 2005). At each time point the percentage of PCV2-infected cells was calculated by counting the total number of cells and the number of PCV2-infected cells and the presence and localization of both PCV2 proteins was determined.

Effect of IFN-γ on the production of PCV2

The influence of IFN-γ on the production of progeny virus in PCV2-infected cells was determined by inoculating PK-15 cells with the standard PCV2-stock. After inoculation, culture medium was added supplemented with 500 U/ml of IFNγ. At 0, 12, 24, 36, 48 and 72 hours post inoculation (hpi) the supernatant was collected. Subsequently, the culture was washed once with PBS. Both the supernatant and the washing fluid were centrifuged for 10 minutes at 15,000 x *g* to pellet cells and debris. The centrifuged supernatant and washing fluids were combined and the extracellular virus was determined. Both pellets and the cell culture were resuspended in PBS and freeze-thawn three times for determination of intracellular virus. Intra- and extracellular virus titres were titrated on PCV-negative PK-15 cells as described previously (Meerts *et al*., 2005) and expressed as titre per 10^5 cells.

Effect of leukocyte-derived porcine cytokines on the total number of PCV2-infected cells

Supernatants of ConA and mock-stimulated PBMCs were added to the culture medium of PK-15 cells after inoculation in concentrations ranging from 6.15 to 50%. After 36 hours of incubation, PK-15 cultures were fixed and stained as described above and the number of PCV2-antigen positive cells was determined.

Statistical analysis

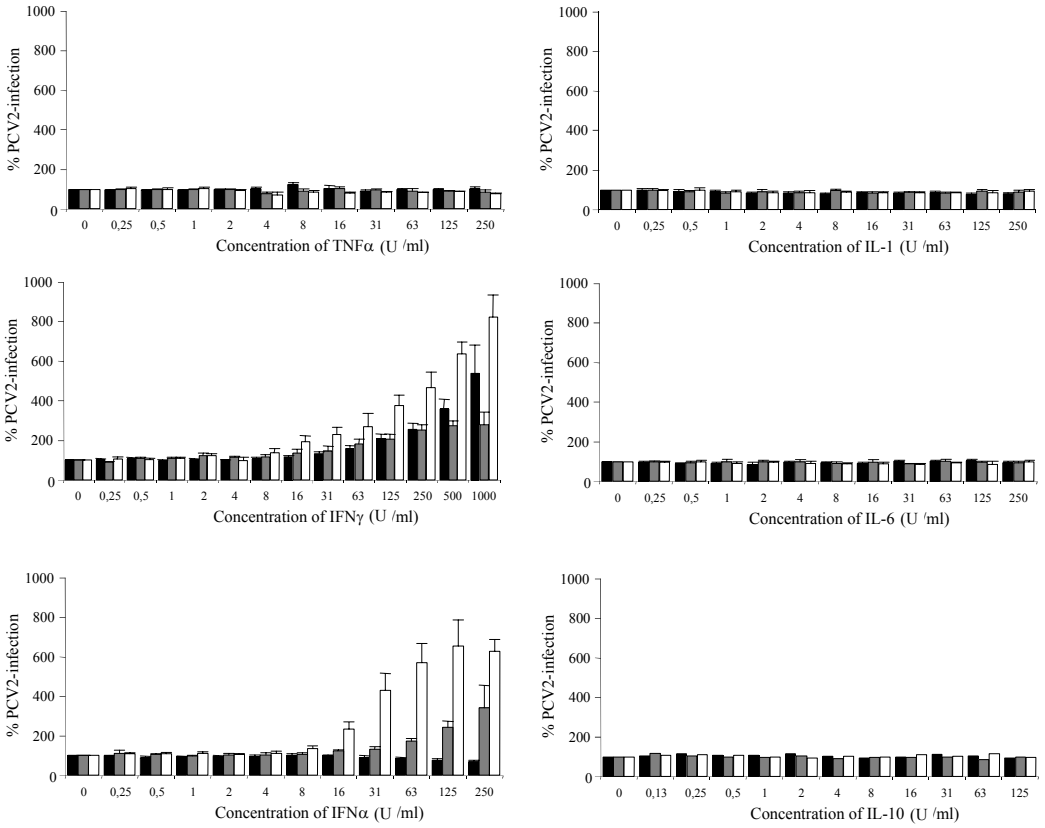
All experiments were repeated three times independently. The results presented in this publication represent the mean values obtained from these three experiments. The variation between different experiments is represented by the standard error of the mean (SEM). Differences were considered to be significant when $p < 0.05$ (p-value calculated with the Mann-Whitney test). Due to limited material, the experiments with IL-10 were performed only once.

Results

Effect of cytokines on the total number of PCV2-infected PK-15 cells

The results of the effects of dilution series of different cytokines on the total number of PCV2-infected PK-15 cells are shown in Figure 1. With TNF-α, IL-1, IL-6 and IL-10, no significant change in the number of PCV2-antigen positive cells was observed at any concentration or at any time of treatment (before, during or after inoculation). Absolute numbers of infected cells in non-treated cultures agreed with previous published data (Meerts *et al*., 2005). With both interferons (IFN-α and IFN-γ) significant changes in the number of PCV2-antigen positive cells were observed.

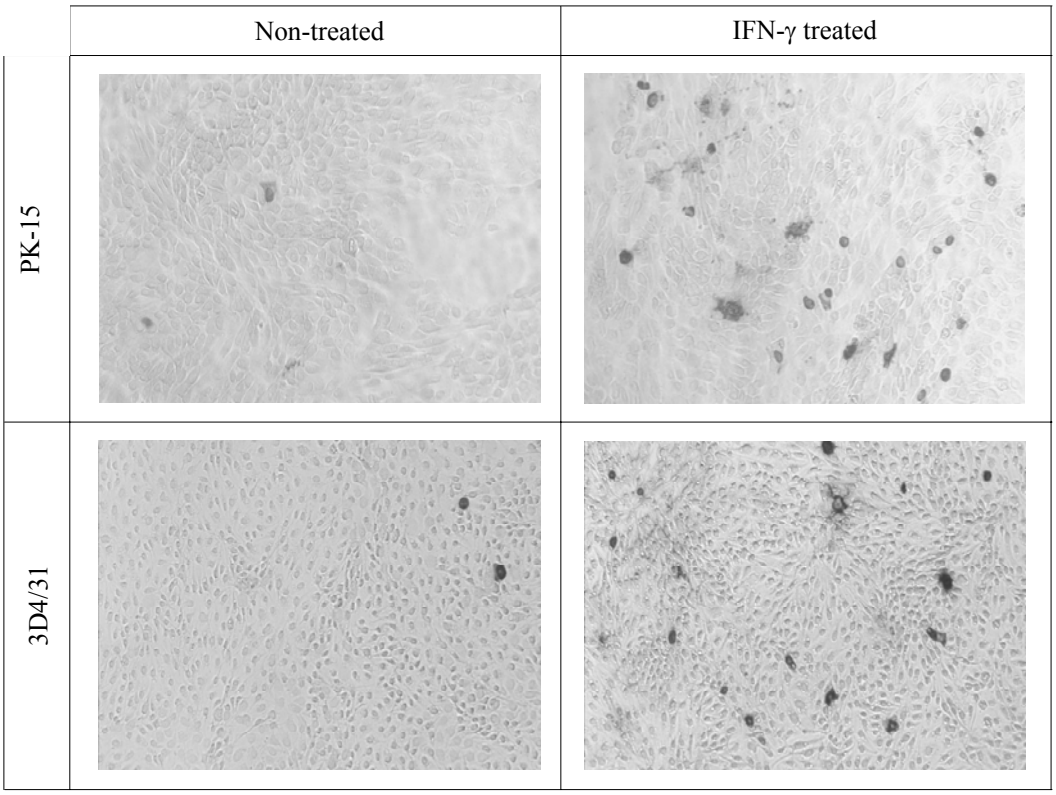
Figure 1. Effect of cytokines on the number of PCV2-infected PK-15 cells. PK-15 cells were treated with recombinant porcine cytokines before (black bar), during (grey bar) or after (white bar) PCV2-inoculation + SEM. The number of PCV2-antigen positive cells in cytokine-treated cells was counted after 36 hours of incubation and compared to the number of PCV2-antigen positive cells in non-treated cells.



IFN-γ induced a dose-dependent increase in the number of PCV2-antigen positive cells regardless of the time point when it was added to the medium of the cells (before, during or after the inoculation). The highest increase was achieved at the highest concentration tested in the experiment (1000 U/ml). When this concentration of IFN-γ was supplemented to the medium before, during or after inoculation, an increase in PCV2-antigen positive cells of 518 ± 134%, 270 ± 57% or 791 ± 105% respectively was observed. The lowest concentrations of IFN-γ that induced a significant increase of PCV2-antigen positive cells when administered before, during or after inoculation were 16, 2 and 2 U/ml respectively. Figure 2 shows a light microscopic picture of PK-15 cells without IFN-γ (negative controls) and with 500 U/ml IFN-γ added in the medium after inoculation. The effects induced by treatment

of cells with IFN-γ could be neutralized when IFN-γ was incubated for 2 hours at 37 °C with IFN-γ neutralizing antibodies (Kontsek *et al*., 1997), prior to incubation with the cells.

Figure 2. Light microscopic pictures of the effect of IFN-γ treatment (500 U/ml) on the number of PCV2 positive cells in PK-15 and 3D4/31 cells. Pictures were taken at a magnification of 100x.



IFN-α induced a significant increase of PCV2-antigen positive cells when administered during or after inoculation, but it induced a significant reduction of infected cells when the cells were pre-treated. At the highest concentration used (250 U/ml) an increase of 341 ± 114% and 629 ± 59% PCV2-infected cells was observed when added respectively during or after inoculation. After pre-treatment, a reduction of 31 ± 6% PCV2-infected cells was observed. The lowest concentrations of IFN-α inducing a significant effect when added before, during or after PCV2-inoculation were 31, 16 and 8 U/ml respectively. The effects induced by treatment of cells with

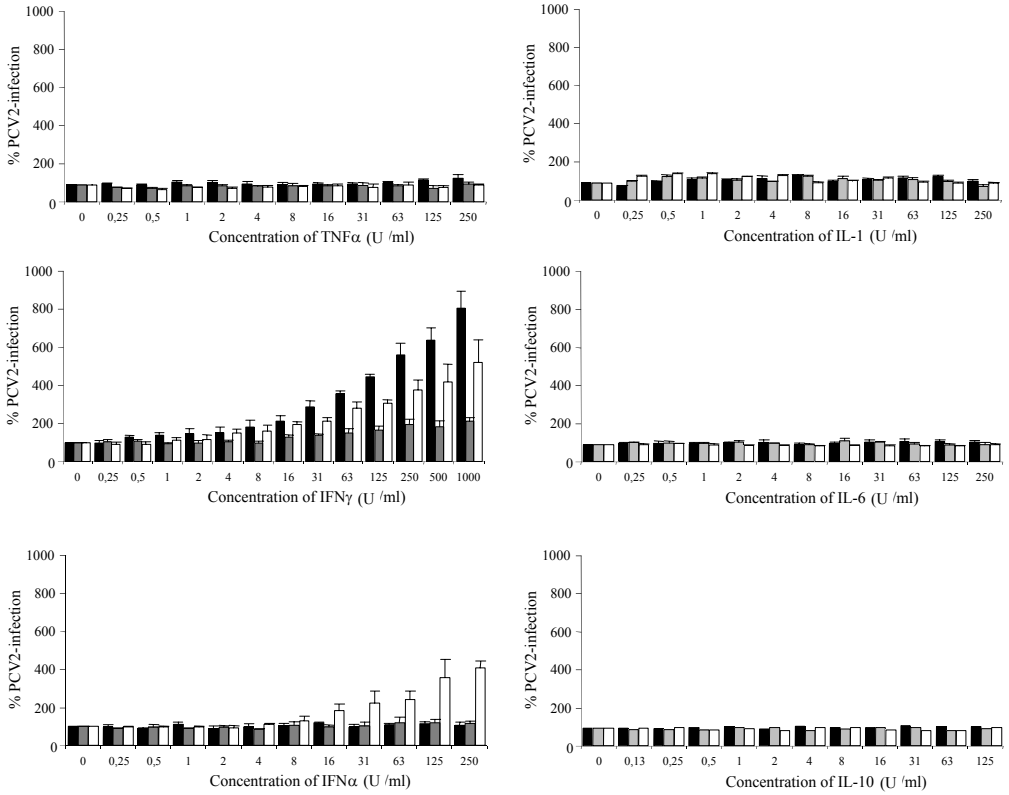
IFN-α could be neutralized when IFN-α was incubated for 1 hour at 37 °C with IFN-α neutralizing antibodies, prior to treatment of the cells.

Effect of cytokines on the total number of PCV2-infected 3D4/31 cells

In 3D4/31 cells, treatment with TNFα, IL-1, IL-6 and IL-10 before, during or after inoculation did not induce a significant change in the number of PCV2-antigen positive cells at any concentration. Similar observations were made in these cells compared to PK-15 cells when the 3D4/31 cells were treated with IFN-α or IFN-γ. IFN-γ induced a dose-dependent increase in the number of PCV2-positive cells when cells were treated before, during or after inoculation. A maximal increase in PCV2-infected cells of 806 ± 88%, 214 ± 16% and 523 ± 115% was observed when cells were treated before, during or after inoculation respectively. When cells were treated with IFN-α before inoculation, no significant changes were observed. When IFN-α was added to the cells during or after inoculation, an increase in infected cells of respectively 115 ± 10% and 408 ± 35% was detected. The results are shown in Figure 3. Figure 2 shows a light microscopic picture of 3D4/31 cells without IFN-γ (negative controls) and with 500 U/ml IFN-γ added in the medium before inoculation.

The effects induced by treatment of cells with IFN-α or IFN-γ could be neutralized when they were incubated for 1 or 2 hours respectively at 37°C with IFN-α or IFN-γ neutralizing antibodies, prior to incubation with the cells.

Figure 3. Effect of cytokines on the number of PCV2-infected 3D4/31 cells. 3D4/31cells were treated with recombinant porcine cytokines before (black bar), during (grey bar) or after (white bar) PCV2-inoculation + SEM. The number of PCV2-antigen positive cells in cytokine-treated cells was counted after 36 hours of incubation and compared to the number of PCV2-antigen positive cells in non-treated cells.

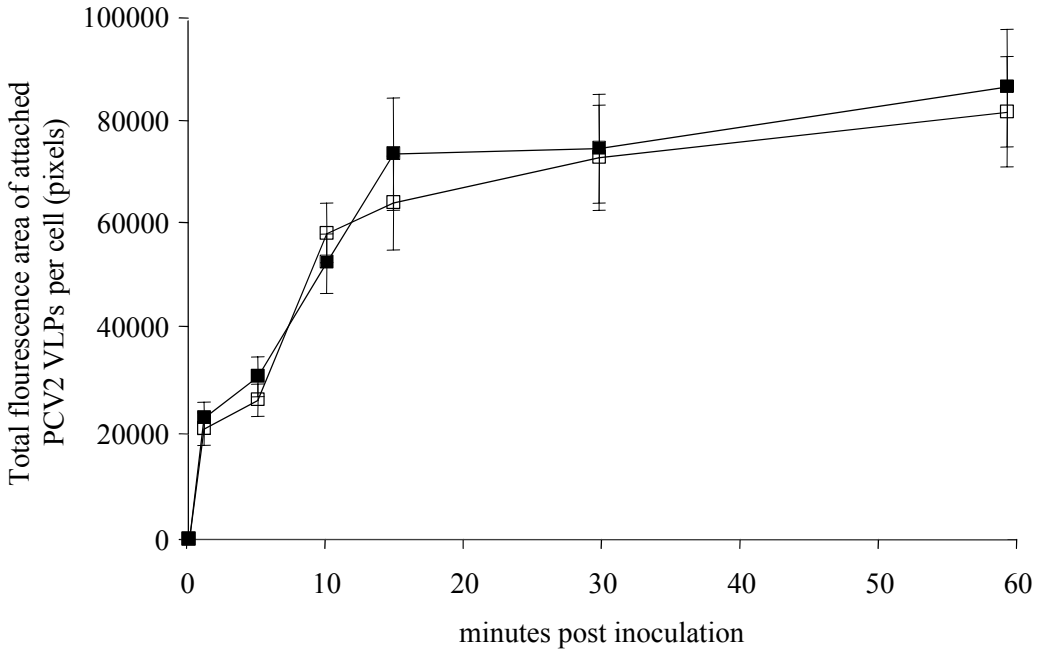


Effect of IFN-γ on attachment and internalization of PCV2 VLPs.

Effect of IFN-γ on attachment of PCV2 VLPs

No significant differences were observed in the number of attached PCV2 VLPs per 3D4/31 cell, nor in the speed of the VLPs binding to 3D4/31 cells treated or non-treated with IFN-γ. The results are shown in figure 4. Both in the IFN-γ treated and non-treated cells, the number of attached PCV2 VLPs per cell increased quickly within 5 minutes and reached a maximum at 15 minutes after incubation. Attached PCV2 VLPs were observed on all cells both in IFN-γ treated and non-treated cultures. Similar observations have been described before (Misinzo *et al*, 2005).

Figure 4. Effect of IFN-γ on attachment of recombinant PCV2 VLPs to 3D4/31 cells The results represent the average number of VLPs attached to 10 cells ± standard deviation in an IFN-γ treated culture (black symbols) and a non-treated culture (empty symbols).



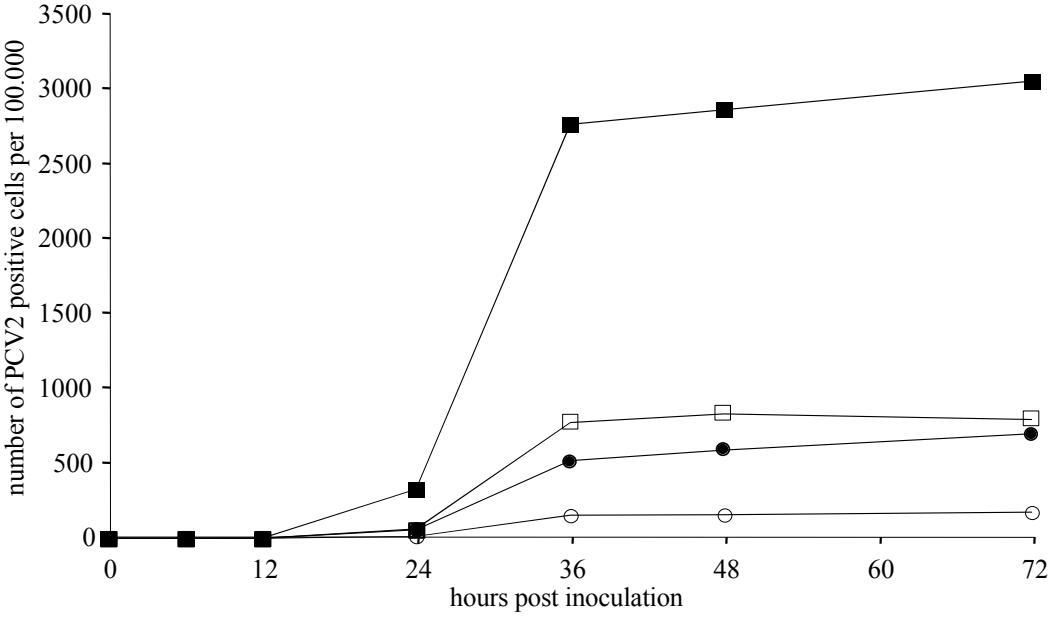
Effect of IFN-γ on entry of PCV2 VLPs

In IFN-γ treated 3D4/31 cell cultures, 4.5% of the cells were found to internalize PCV2 VLPs at 30 minutes after incubation with the VLPs compared to 2.5% of the cells in non-treated cultures. PCV2 VLP internalizing cells in IFN-γ treated cultures, contained more VLPs inside the actin rim (18.7 ± 10.1%) compared to internalizing cells in non-treated cultures (4.0 ± 3.0%).

Effect of IFN-γ on PCV2 protein expression kinetics

When the evolution in the number of capsid and Rep protein positive cells was compared in IFN-γ treated and non-treated PK-15 cultures, similar kinetics were observed in both cultures. The only difference was that in IFN-γ treated cultures approximately 5 times more cells were positive for the capsid or Rep protein compared to the non-treated cultures. No differences were observed in the time point at which the first capsid or Rep proteins could be detected. The fraction of capsid protein positive cells against the Rep protein positive cells was not altered by treatment of the cell cultures with IFN-γ. Figure 5 shows the evolution in the number of PK-15 cells expressing PCV2 capsid protein and Rep protein in PK-15 cultures with or without IFN-γ treatment.

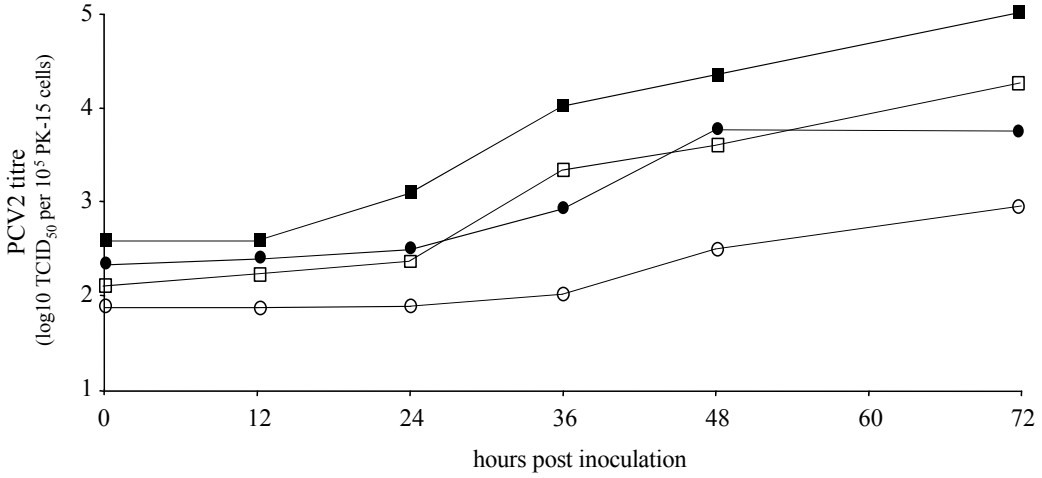
Figure 5. Evolution in the number of cells expressing capsid (squares) or Rep protein (circles) in IFN-γ treated (black symbols) and non-treated (empty symbols) PK-15 cultures



Effect of IFN-γ on the production of PCV2

Since IFN-γ increased the number of PCV2-antigen positive PK-15 cells independently of the time point when it was added to the medium of the cells, this cytokine was selected to investigate the effect on the production of progeny virus at a concentration of 500 U/ml. The results of the production experiment are presented in Figure 6. Both extracellular and intracellular virus production were increased in the IFNγ-treated cells compared to the non-treated control cells. A significant higher titre in IFN-γ treated cells could be demonstrated starting from 36 hpi. At 72 hpi, the intracellular and extracellular PCV2 titres in the non-treated culture were 3.7 ± 0.3 $\log_{10}$ $TCID_{50}$ and 2.9 ± 0.6 $\log_{10}$ $TCID_{50}$ per 10^5 cells respectively while in IFN-γ treated cultures intracellular and extracellular PCV2 titres were 5.0 ± 0.3 $\log_{10}$ $TCID_{50}$ and 4.2 ± 0.4 $\log_{10}$ $TCID_{50}$ per 10^5 cells. IFN-γ treatment increased both intracellular and extracellular PCV2 titres 20 times (1.3 $\log_{10}$).

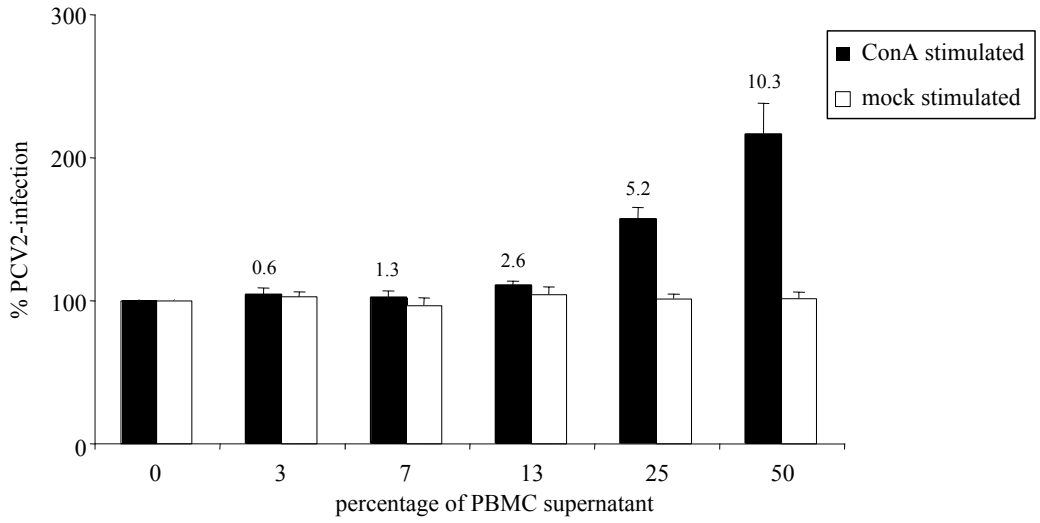
Figure 6. Kinetics of intracellular (squares) and extracellular (circles) PCV2 titres in PK-15 cells treated with 500 U/ml IFN-γ (black symbols) compared to non-treated (empty symbols) cells.



Effect of leukocyte-derived porcine cytokines on the total number of PCV2-antigen positive cells

The results of the incubation of PK-15 cells with the leukocyte-derived porcine cytokines are shown in Figure 7. The supernatant of ConA-stimulated PBMCs caused a dose-dependent increase in PCV2-antigen positive cells when it was added to the medium of PCV2-inoculated PK-15 cells. When a concentration of 50% of this supernatant was added to the cells, an increase of 215 ± 22% was observed. Again the supernatant of non-stimulated PBMCs did not influence the number of PCV2-infected cells. Incubation of the supernatants with IFN-γ neutralizing monoclonal antibodies prior to treatment of PK-15 cells, but not with IFN-α neutralizing antibodies, inhibited the increased infection of PCV2.

Figure 7. Effect of supernatant of ConA and mock-stimulated PBMCs on the number of PCV2-infected PK-15 cells + SEM. IFN-γ concentrations (U/ml) in dilutions of ConA stimulated PBMC supernatants are shown on top of the corresponding bars.



Discussion

In the present study, it was shown that type I and type II interferons are able to enhance PCV2-infection and production in PK-15 and 3D4/31 cells. This is a very important finding to understand the interaction of circoviruses with their host cell but also in view of the pathogenesis and reproduction of postweaning multisystemic wasting syndrome in PCV2-infected pigs.

Interferons are mainly known for their antiviral activity. This antiviral effect has been demonstrated for many viruses and has been found to be so consistent and potent that recombinant interferons have been commercialized to control viral infections in humans and animals (Davis, 1997; Martin *et al*., 2002). To our knowledge, the present study is the first study to show an enhancement of a viral infection by both type I and type II interferons. Previously virus-enhancing properties were already attributed to IFN-γ with relation to the cell-mediated transmission of Human Immunodeficiency Virus (HIV) (Dianzani *et al*., 1996). Both interferons (IFN-α and IFN-γ) included in the present study were able to increase PCV2-infection in continuous porcine cell lines under specific conditions. IFN-γ was found to have the most consistent pro-viral effect on PCV2 in PK-15 and 3D4/31 cells independent of the time point of exposure. In contrast, IFN-α treatment of the cells had a pro-viral effect on PCV2 only when used during or after inoculation and had a mild but significant antiviral effect in PK-15 when used before inoculation. Since IFN-γ induced the most consistent increase of PCV2-infection (independent on the time of treatment), this work further focussed on the effect of this cytokine on the interaction between PCV2 and its target cell. The effect of recombinant IFN-γ on PCV2-infection in PK-15 cells was confirmed by using leukocyte-derived cytokines. The PCV2-infection enhancing effect of supernatant of ConA-stimulated PBMCs could be completely inhibited by IFN-γ neutralizing antibodies, indicating that IFN-γ was indeed the factor responsible for the enhancement of the infection. IFN-γ treatment rendered PK-15 cell cultures more susceptible to PCV2 infection. When the same PCV2-stock was titrated on IFN-γ pre-treated (500 U/ml) or non-treated PK-15 cells, a 10 times higher titre was obtained in the treated cell cultures (data not shown). This shows that the sensitivity of the classical PCV2-titration assay on PK-15 cells can be increased at least 10 times by IFN-γ treatment.

In the present study, a first step was made to understand the influence of IFN-γ on PCV2-infection *in vitro*. Different steps of the virus-cell interaction were analyzed in IFN-γ treated and non-treated cells. It was observed that the increased infection of IFN-γ treated cells was not induced by an increased binding of the virus to the cell surface, but by an increased internalization of bound virions into the cell. Since no increased binding of PCV2 VLPs to the cell membrane was observed, it could be concluded that IFN-γ treatment did not increase the expression of the PCV2-receptor(s) on the cell membrane. The increased internalization of the virus into the cell was most probably induced by an influence on a further step in the internalization cascade. It looks like IFN-γ is able to activate or increase cytoplasmic or membrane-spanning factors that play a role in the endocytosis of the virus-receptor complex. Further research is necessary to identify the exact mechanism involved. Once inside the cell, the viral genome should be transported towards the nucleus of the cell. Influences of IFN-γ on the intracellular trafficking of PCV2 and the nuclear import of its genomic material have not been investigated in the current study and remain possible. Both known PCV2 proteins could be detected for the first time at the same time points in IFN-γ treated or non-treated cultures. The only difference that could be observed upon IFN-γ treatment (500 U/ml) was that a higher number of cells contained these proteins. Progeny virus was detected starting from the same time point in both cultures. In IFN-γ treated cultures, 20 times more progeny virus was detected, indicating that the increased progeny virus yield was not only due to a higher number of infected cells, but also due to a higher production of progeny virus per infected cell. For another virus with a circular ssDNA genome that uses the same DNA replication mechanism (phage ϕX), it has already been shown that the amount of produced progeny virus is correlated with the number of early formed double stranded DNA replication forms and thus with the number of virions infecting the cell (Koths *et al.*, 1980). A similar mechanism might be involved in PCV2-replication since PCV2 VLP internalizing cells in IFN-γ treated cultures contained approximately 5 times more VLPs inside their cell membrane compared to internalizing cells in non-treated cultures.

When IFN-α or IFN-γ was added to PK-15 cells both before and after inoculation, IFN-γ increased whereas IFN-α reduced the number of infected cells (data not shown). This combination of pre and post-treatment resembles best the

effect that can be expected *in vivo*. When the data obtained in this *in vitro* study are extrapolated to the *in vivo* situation, the most important PCV2-enhancing effect in this situation can be expected from IFN-γ. The present observations have important consequences on the current PCV2 research with relation to postweaning multisystemic wasting syndrome. IFN-γ is generally produced during an immune response. If the interferon-induced increase of PCV2-replication observed *in vitro,* also takes place *in vivo*, this might be the common event in all studies that have shown to activate the replication of PCV2 in animals by inducing immune stimulations (Allan *et al*., 2000a; Rovira *et al*., 2002; Allan *et al*., 2000b, Krakowka *et al*., 2001). The observations made in this study may also explain why gnotobiotic pigs are more resistant to PCV2-infection and disease. Since gnotobiotic pigs do not encounter regular immunological stimulations, PCV2 might not have an ideal environment created by type I or type II interferons to replicate to high levels.

PCV2 is highly related to PCV1, another porcine circovirus that has not been associated with any disease. It was quite interesting to see that type I and type II interferons did not influence PCV1-infection in PK-15 cells (data not shown). The question can be asked if the influence of interferon on infection is involved in the higher virulence of PCV2 compared to PCV1 *in vivo*. The mechanism used by PCV2 to profit from type I or type II interferons produced in response to other infections, may also be important for other related viruses. TT-virus (TTV) is also a member of the *circoviridae* (Nishizama *et al*., 1997). It is a small human-infecting virus with a circular ssDNA genome that is widely spread in the population. In literature, indications are already present that promotion of TTV-infection and replication might occur through a similar mechanism. It was demonstrated that human PBMCs were able to sustain productive TTV infection only when they were stimulated with a combination of lipopolysaccharide, IL-2 and phytohemagglutinin (PHA) (Mariscal *et al*., 2002). Similar to ConA, PHA is a mitogen that, besides other activities, stimulates T-cells to proliferate and produce IFN-γ amongst other cytokines (Verfailly et al., 2001). The role of IFN-γ was not investigated in that study. TTV is in some cases associated with hepatitis. Another important virus causing hepatitis is the hepatitis C virus (HCV). Both viruses are often found to be present at the same time in affected patients. A common treatment of HCV-infected patients is the administration of recombinant human IFNα (Maggi *et al*., 2001). Since it has been shown in the present

study that the closely related PCV2 is able to profit from interferon treatment, it is important that the interaction between TTV and type I and type II interferons is also investigated.

The effect of type I and type II interferons on PCV2-infection offers many possibilities to better understand the pathogenesis of PCV2 and possibly also of other circoviruses. Further research will therefore focus on the cell-biological effects of interferons with regard to circovirus infection and on the importance of this mechanism in the induction of PMWS in the natural host.

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4.2.

Increased porcine circovirus type 2 replication in gnotobiotic pigs treated with concanavalin A

Manuscript in preparation

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Summary

Previously, it has been shown that interferon-gamma (IFN-γ) enhances porcine circovirus type 2 (PCV2)-replication *in vitro*. In the present study, it was investigated if the PCV2-enhancing properties of IFN-γ, could also be demonstrated in the natural host of PCV2. Gnotobiotic pigs were injected with Concanavalin A (ConA) or recombinant IFN-γ (rIFN-γ) 12 hours before PCV2-inoculation and afterwards every 3 days during a period of 9 days. The replication of PCV2 was monitored at 3 different time points in individual pigs by taking lymph node biopsies at 10 and 15 days post inoculation (dpi) and by collecting the remaining parts of the lympn nodes at euthanasia at 21 dpi. In non-treated control pigs and rIFN-γ inoculated animals, PCV2 could be detected starting from 15 dpi. No significant differences in the level of PCV2-replication between pigs from these different groups were found. In gnotobiotic pigs treated with ConA, PCV2 could already be isolated from inguinal lymph nodes starting from 10 dpi. At 15 dpi, the level of PCV2-replication was significantly higher in ConA-treated pigs compared to the pigs of other groups. Although ConA treatment significantly increased PCV2-replication, no signs of disease were observed in any of the inoculated or non-inoculated animals. These experiments indicate that ConA-treatment enhances the replication of PCV2 *in vivo*, however it could not be demonstrated that this effect was IFN-γ dependent.

Introduction

Porcine circoviruses are very small and relatively simple viruses (Tischer *et al*., 1982). Until present, only 3 different viral proteins have been described that are being expressed in porcine circovirus-infected cells (Morozov *et al.*, 1998; Mankertz and Hillebrand, 2001). It is not hard to accept that this limited array of viral proteins makes the virus incapable of intensively manipulating the environment in its host in favour of its own replication. As far as it is known today, circoviruses do not possess proteins that for instance are able to modify the immune response of their host, as other more complex viruses do (Tortorella *et al.*, 2000). This might account for the observation that the replication of porcine circovirus 2 (PCV2) is intensively influenced by host specific, as well as by external factors. Pigs with identical environmental backgrounds are not equally able to cope with a PCV2-infection, which is an indication for a genetical base of their susceptibility (Ladekjær-Mikkelsen *et al*., 2002; Sanchez *et al*., 2003; Meerts *et al*., 2005a). The impact of the environment on the susceptibility of the pigs to the infection and subsequently to its clinical outcome, has also been documented. Coinfections with other agents may have important effects on the clinical outcome of a PCV2-infection in field conditions. Porcine parvovirus, porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma Hyopneumponiae* infections in PCV2-inoculated pigs all enhance the replication of PCV2 and exacerbate the clinical outcome of the infection (Allan *et al*., 2000a; Allan *et al*., 2000b; Rovira *et al*. 2002; Opriessnig *et al*., 2004). Injection of Keyhole limpet hemocyanin (KLH) in PCV2-inoculated gnotobiotic pigs suggested that the induction of an immune response might be the key factor in the induction of the increased PCV2-replication although this increased PCV2-replication could not be reproduced in conventional SPF pigs (Krakowka *et al*., 2001; Ladekjær-Mikkelsen *et al*., 2002). A mechanism by which an immune response against another infectious agent would be able to enhance the replication of PCV2 remains unidentified.

Recently, it was shown that interferon-alpha (IFN-α) and interferon-gamma (IFN-γ) are able to enhance PCV2-replication *in vitro* when administered under certain conditions. Especially IFN-γ was effective in enhancing PCV2 replication (Meerts *et al*., 2005c). IFN-γ is a Th1-specific cytokine that is generally produced

during the onset of a cellular immune response. It has been demonstrated that PRRSV-infections (Batista *et al*., 2004), parvovirus infections (Kerr *et al*., 2004) and the injection of KLH (Connor *et al*., 2001), all lead to the production of IFN-γ as a component of the initiation of the immune response. Furthermore, vaccine adjuvants are being composed in a way that they elicit a better cellular immune response against the immunogen. This way they initiate a higher production of IFN-γ (Avramidis *et al*., 2002). Previously, it has also been shown that vaccine adjuvants are able to enhance the level and duration of PCV2-replication in pigs and exacerbate the clinical outcome of the infection (Opriessnig *et al*., 2003). In the studies mentioned above, the IFN-γ response was never monitored.

It was the aim of the present study to investigate if IFN-γ also enhances PCV2-infection *in vivo* as was observed *in vitro*.

Materials and methods

Virus, recombinant IFN-γ and Concanavalin A

In the present study, The PCV2 strain 1121 was used (Meehan *et al*., 2000) at a fourth passage level on PK-15 cells. Recombinant IFN-γ (rIFN-γ) was purchased from R&D systems (Abingdon, UK), Concanavalin A (ConA) was purchased from Sigma (Heidelberg, Germany). ConA is a lectin that induces proliferation of Th1 cells. It will stimulate Th1 cells to produce high concentrations of Th1-specific cytokines amongst which IFN-γ both *in vitro* (Verfailly *et al*., 2001) and *in vivo* (Tiegs, 1997).

Pigs and experimental design

Seventeen gnotobiotic pigs were derived by caesarian section from 2 Landrace sows. They were raised in sterile conditions in individual isolators. Pigs were randomly divided in 5 groups. An overview of these groups and their specific treatments is given in Table 1.

Table 1. Distribution of pigs in different groups with specific manipulations per group

Group	n	PCV2-inoculation	Treatment	Pig numbers
A	4	yes	-	1 to 4
B	4	yes	ConA	5 to 8
C	4	yes	rIFN-γ	9 to 12
D	3	no	ConA	13 to 14
E	2	no	rIFN-γ	15 to 16

At 19 days of age, all pigs from groups A (PCV2/-), B (PCV2/ConA) and C (PCV2/IFN-γ) were inoculated intraperitoneally and oronasally with a total dose of $10^{4.3}$ $TCID_{50}$ of PCV2 strain 1121 propagated in PK-15 cells. This route of inoculation was chosen to obtain a reproducible infection in all pigs without interference of the primary site of replication. Pigs of groups D (-/IFN-γ) and E (-/ConA) were mock-inoculated with equal volumes of cell culture medium.

Pigs from groups C (PCV2/IFN-γ) and D (-/IFN-γ) were injected every three days with rIFN-γ at a dose of 100,000 units (3 µg of recombinant protein) per treatment: 50,000 units were injected intramuscular and 50,000 units were injected subcutaneous in the lower abdomen. The first injection was administered 12 hours before the inoculation of the pigs and repeated at 2, 5 and 8 days post inoculation (dpi).

Pigs from groups B (PCV2/ConA) and E (-/ConA) were treated at the same time points with ConA at a dose of 1.5 mg/kg bodyweight. Even lower doses were found to induce a significant IFN-γ production in mice (Miyagi *et al.*, 2004). Half the dose was injected intramusculary and the other half was injected subcunateous in the lower abdomen.

Pigs were monitored for a period of 21 days after PCV2-inoculation and euthanized afterwards.

Clinical monitoring and sampling

Pigs were monitored daily for the appearance of clinical signs that are associated with the postweaning multisystemic wasting syndrome (PMWS). These clinical signs

include cachexia, depression, respiratory distress and jaundice. Plasma was collected from all pigs at 0, 7, 10, 15 and 21 dpi to monitor the production of anti-PCV2 antibodies as well as PCV2-neutralizing antibodies.

In order to follow the degree of PCV2-replication in all individual pigs, inguinal lymph node biopsies were collected at different time points as described before (Meerts *et al*., 2005). At 10 days post inoculation (dpi) a biopsy was taken from the left inguinal lymph node and at 15 dpi a biopsy was taken from the right inguinal lymph node. In order to take lymph node biopsies, the pigs were anaesthetized by intramuscular injection of 2.2 mg tiletamine and 2.2 mg zolazepam (Zoletil®, Virbac) dissolved in 0.22 ml 2% Xylazine (XYL-M® 2%, VMD) per kilogram body weight. An incision was made in the overlaying skin and half the inguinal lymph node was excised. The wound was closed by mersilene sutures (Mersutures®, Ethicon). At the time of euthanasia (21 dpi) the remaining parts of both left and right inguinal lymph nodes were collected. Ten % suspensions were made from the biopsies and the lymph nodes and the level of PCV2-replication in these organs was determined by PCV2-titration on PK-15 cells. The detection limit of this technique was $10^{2.4}$ $TCID_{50}$/g.

Analysis of the humoral immune response against PCV2

Plasma samples were used to detect the total anti-PCV2 antibody titre by an immunoperoxidase monolayer assay (IPMA) described previously (Labarque *et al*., 2000). PCV2-neutralizing antibodies were determined by calculating the percentage of inhibition of PCV2-infection by the sensitive seroneutralization assay (sensitive SN) (Meerts *et al*., 2005b). Briefly, a standard infectious dose of PCV2 ($10^{3.7}$ $TCID_{50}$/ml) was incubated for 1 hour at 37°C with 25% of the serum. Afterwards, this virus-serum mixture was inoculated on PCV-negative PK-15 cells. After 1 hour, the inoculum was removed, the cell cultures were washed 2 times, new cell medium was added and cultures were maintained at 37°C in the presence of 5% CO2. After 36 hours of incubation, after the first replication cycle of PCV2 in PK-15 cells was completed, the cells were fixed and stained for PCV2 antigens using an immunoperoxidase technique. The number of PCV2-infected cells was counted and compared to the number of infected cells in a PK-15 culture inoculated with a mock-treated stock. The result of this seroneutralization assay is expressed as a percentage of reduction of PCV2-infection.

Statistical analysis

The statistical significance of differences between medians of different groups was tested by applying two-sided Mann-Whitney tests. Differences were considered significant when $p < 0.05$.

Results

Clinical monitoring

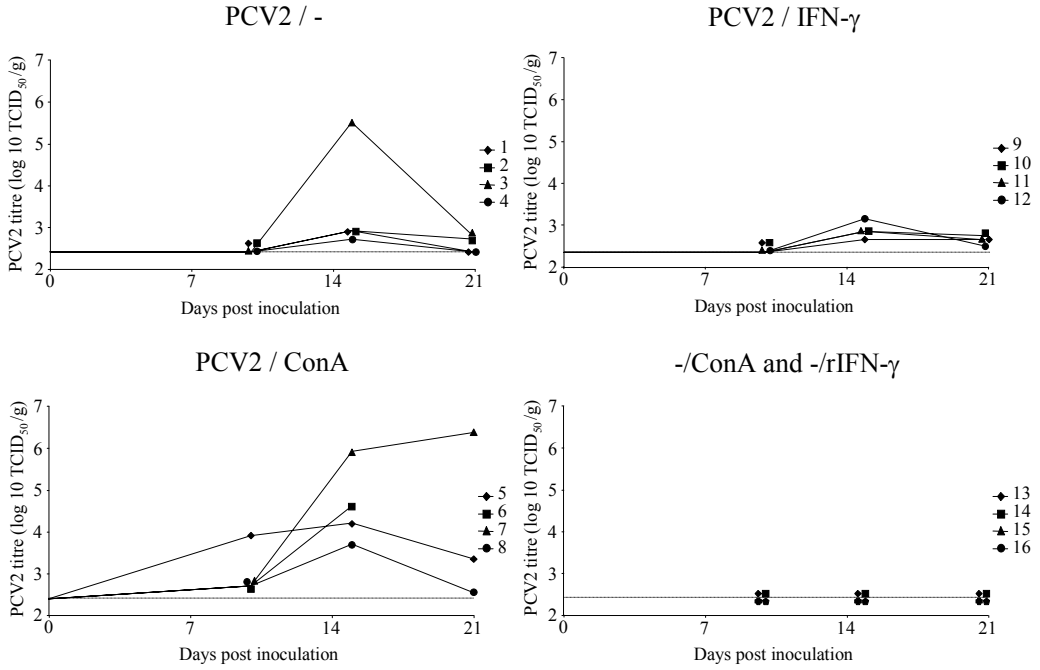
During the study, clinical signs suggestive of PMWS were not observed in any of the pigs. Pig 6 (PCV2/ConA) died during blood sampling at 15 dpi. At the time of euthanasia, no differences in general appearance were observed between PCV2-inoculated and non-inoculated pigs or between pigs of groups that received different treatments. Starting from the first time when a biopsy was taken, up to the end of the study, it was observed that inguinal lymph nodes of pigs treated with rIFN-γ or ConA, were noticeably enlarged and pale compared to the lymph nodes of non-treated pigs. This enlargement of the inguinal lymph nodes was independent of the PCV2-inoculation.

PCV2 replication

The evolution of PCV2 replication in the inguinal lymph nodes of individual pigs is shown in Figure 1. In pigs of groups D and E (non-inoculated pigs), PCV2 was not isolated at any time point during the study. From all PCV2-inoculated pigs, PCV2 was isolated at at least one time point. PCV2 could be detected for the first time at 15 dpi in pigs of groups A (PCV2/-) and C (PCV2/IFN-γ). In pigs of group B (PCV2/ConA), PCV2 was already isolated from the lymph node biopsies taken at 10 dpi. Titres at this time point ranged between $10^{3.1}$ and $10^{4.3}$ $TCID_{50}$/g. At 15 and 21 dpi, PCV2 titres in ConA-treated pigs were generally higher than titres at the same time point in other pigs but due to the high variation in PCV2-titres between pigs in the same groups and due to the limited number of pigs, a significant difference could only be demonstrated between the mean titres of groups B (PCV2/ConA) and C (PCV2/IFN-γ) at 15 dpi.

The general evolution of PCV2-replication in time was similar in all pigs except one. Eleven out of twelve PCV2-inoculated pigs showed a peak of PCV2-replication at 15 dpi. Afterwards the PCV2-titres decreased in all these pigs. However pig 7 (PCV2/ConA) showed a gradual increase in PCV2 titre until the end of the study.

Figure 1. Evolution of PCV2-replication in inguinal lymph node biopsies of PCV2-inoculated gnotobiotic pigs, non-treated or treated with Concanavalin A (ConA) or recombinant Interferon-gamma (rIFN-γ). The dashed line represents the detection limit of the assay ($10^{2.4}$ $TCID_{50}$/g).

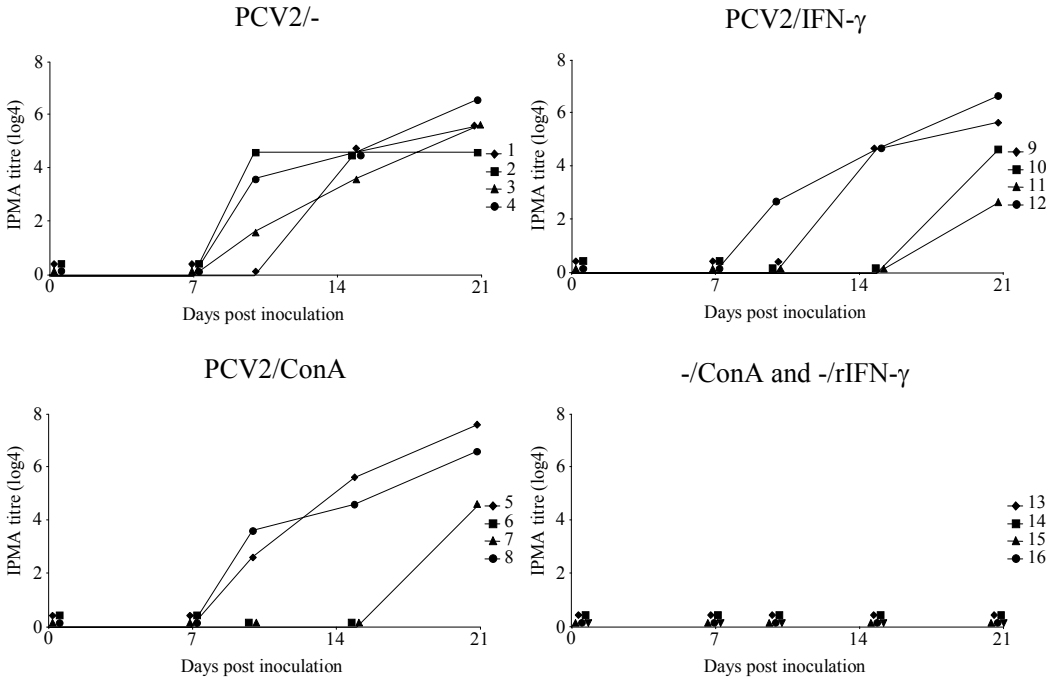


Anti-PCV2 antibodies (IPMA)

The evolution in IPMA antibodies against PCV2 is shown in Figure 2. Seroconversion against PCV2 was not detected in any of the pigs from groups D or E (non-inoculated pigs). Anti-PCV2 antibodies (IPMA antibodies) were demonstrated in all PCV2-inoculated pigs except the pig that died at 15 dpi 6 (PCV2/ConA). Differences were observed in the time point at which anti-PCV2 IPMA antibodies were detected for the first time. Pigs from group A (PCV2/-) seroconverted at 10 or

15 dpi. In Groups B (PCV2/ConA) and C (PCV2/IFN-γ), only 2 out of 4 pigs showed IPMA antibodies against PCV2 at 15 dpi. In the other pigs, seroconversion against the virus was delayed until 21 dpi. During the study, no significant differences could be detected between the mean IPMA antibody titres of different groups.

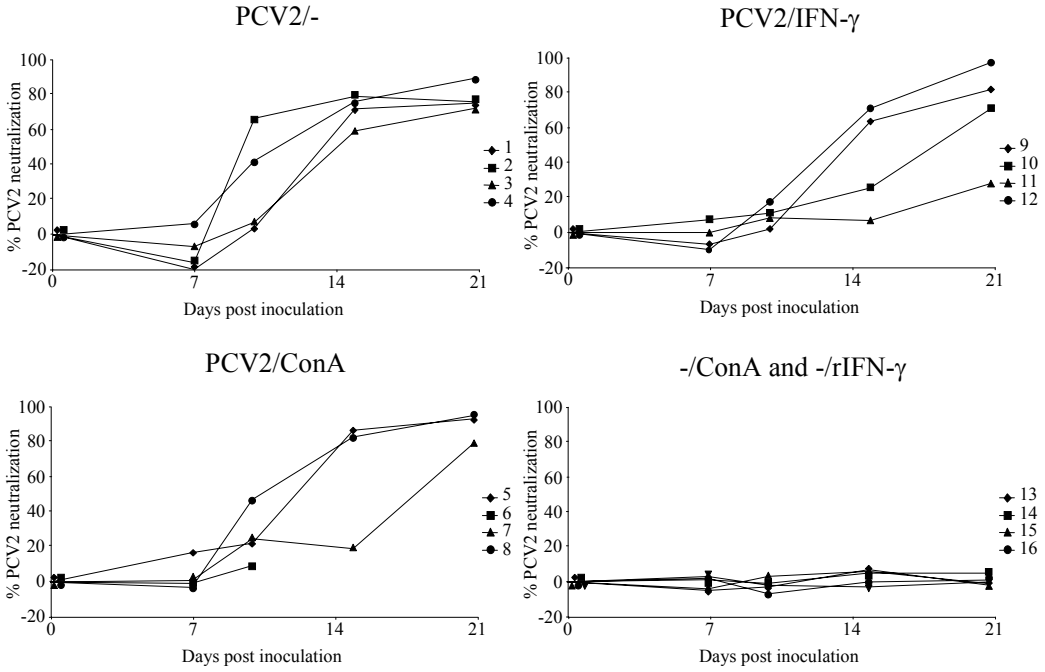
Figure 2. Evolution of anti-PCV2 antibody titres (IPMA) in PCV2-inoculated gnotobiotic pigs, non-treated or treated with Concanavalin A (ConA) or recombinant Interferon-gamma (rIFN-γ).



PCV2-neutralizing antibodies (sensitive SN)

PCV2-neutralizing antibodies generally followed the same evolution compared to the IPMA antibodies. Their evolution in individual pigs can be followed in Figure 3. Neutralizing antibodies were detected for the first time at 10 dpi in pigs of group A (PCV2/-). Pigs in groups B (PCV2/ConA) and C (PCV2/IFN-γ) that showed a delayed production of IPMA antibodies, also showed a delayed production of PCV2-neutralizing antibodies. Again, no statistical differences were demonstrated between the SN titres at 21 dpi between different groups.

Figure 3. Evolution of PCV2-neutralizing antibody titres (SN) in PCV2-inoculated gnotobiotic pigs, non-treated or treated with Concanavalin A (ConA) or recombinant Interferon-gamma (rIFN-γ).



Discussion

In the present study, it was shown that treatment of gnotobiotic pigs with Concanavalin A (ConA) rendered the pigs more susceptible to an increased replication of PCV2. This increased PCV2-replication could not be induced by injecting the pigs with recombinant IFN-γ alone, following the protocol used in the present study.

The different outcome of the ConA and rIFN-γ treatment can be due to various mechanisms. The first mechanism that might be responsible for the difference between the ConA-treated and the rIFN-γ injected animals is that ConA-treatment induces much higher concentrations and a more prolonged production of IFN-γ in animals compared to the IFN-γ concentration that can be reached by injecting the recombinant form of IFN-γ in the pig. It has been demonstrated that rIFN-γ, injected in an animal is very quickly removed from the body (Lortat-Jacob *et al*., 1996) while ConA induces a continuous production of IFN-γ by the T-cells of the animal itself. A preliminary study in conventional pigs showed that after rIFN-γ injection, a peak in serum concentration of IFN-γ was reached at 12 hours after injection. The concentration of IFN-γ rapidly decreased afterwards and could no longer be detected at 24 hours after injection. ConA treatment induced concentrations of IFN-γ in serum up to 4 times higher which were detectable for several days (data not shown). Using the same newly developed and not yet validated technique, IFN-γ could not be detected in the serum of the gnotobiotic pigs in the present study, indicating that the levels of IFN-γ circulating in the blood were very low. This could be caused by efficient binding of the cytokine to its receptors. The treatment of pigs with rIFN-γ or ConA were clearly effective since inguinal lymph nodes of treated pigs were clearly enlarged. Secondly, it is known that ConA, by stimulating Th1 cells to proliferate, also induces the production of other cytokines and alters the state of the T-cells. These changes might also have an influence on the pig's susceptibility to PCV2. The results from the present study are indicative that the influence of ConA treatment on PCV2-replication was not merely due to IFN-γ alone. More research needs to be performed to settle this issue.

One of the common effects that was observed by rIFN-γ injection and ConA treatment was the effect on the onset of the humoral immune response against PCV2.

Two out of four pigs in each group (IFN-γ injected and ConA-treated) showed a delayed onset of the humoral immune response against PCV2. Such an effect of ConA or IFN-γ has not been demonstrated *in vivo* before. A possible mechanism responsible for this effect is the inhibitory effect of IFN-γ on the early stages of activation or homing of B-cells (Abed *et al*., 1994; Ben Jilani *et al*., 2001; Flaishon *et al*., 2001).

In the present study, it was again demonstrated that gnotobiotic pigs are very resistant to PCV2-induced disease. Even in the ConA-treated pigs that experienced an increased PCV2-replication, no clinical signs were observed. Still, the ConA-treatment might provide scientists with a valuable tool to investigate the pathogenesis of PMWS and more precisely the link between the replication of the virus and the disease since it is quite cheap and easy to perform. Several treatments of gnotobiotic pigs have been described until now. When these techniques are compared, we come to a remarkable result. It has been demonstrated that an immune-stimulation (Krakowka *et al*. 2001) or mimicking a part of the immune response (the present study), is able to increase PCV2 replication in pigs. However, an artificial immune suppression induced by Cyclosporin A (CysA) treatment resulted in a similar effect on the replication of the virus in its host (Krakowka *et al*., 2003; Meerts *et al*., 2005). Thus, activating T-cells (ConA) as well as blocking T-cells (CysA) enhances PCV2-replication in the pig. The difference in virological outcome between these two treatments was that CysA-treatment induced a higher replication of the virus but was not able to expedite the replication, whereas ConA-treatment induced both. Probably the effect induced by CysA is merely due to the inhibition of the specific immune response while ConA causes a PCV2-replication enhancing effect. This observation indicates that there is an unstable equilibrium between a specific immune response that is able to neutralize PCV2 and PCV2-infected cells and an immune response that facilitates the replication of the virus in the pig. This fragile balance might be the underlying mechanism responsible for the high variation that is observed in PCV2-replication and clinical outcome between individual pigs and the even higher variation between different studies.

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5.

Correlation between the immune response and the level of porcine circovirus type 2 replication

5.1. CORRELATION BETWEEN TYPE OF IMMUNE RESPONSE AGAINST PORCINE CIRCOVIRUS TYPE 2 AND LEVEL OF VIRUS REPLICATION

5.2. ABSENCE OF PORCINE CIRCOVIRUS TYPE 2 NEUTRALIZING ANTIBODIES IN PIGS WITH POSTWEANING MULTISYSTEMIC WASTING SYNDROME

5.1.

Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication

Viral Immunology (2005) 18, 333-341

P. Meerts, S. Van Gucht, E. Cox, A. Vandebosch and H. J. Nauwynck

Summary

PCV2-replication is characterized by a high variation between infected pigs. This study investigated the role of immunologic responses in causing this variation. Twelve gnotobiotic pigs were inoculated with PCV2. Four of these pigs were treated with Cyclosporin A (CysA) to monitor the effect of the adaptive immunity on the development of the PCV2-infection. Through lymph node biopsies at 10, 15 and 21 days post inoculation (dpi), PCV2-replication in lymphoid tissues was monitored. The production of total PCV2-specific and PCV2-neutralizing antibodies was followed, together with interferon-gamma (IFN-γ) mRNA expression levels in peripheral blood monocytes as a marker for cellular immunity. Generally, the CysA-treated pigs showed the highest PCV2-titres, indicating that the adaptive immunity is necessary to restrain PCV2-replication. Three different PCV2-replication patterns were observed in non-CysA-treated pigs. Pattern 1: in two pigs, PCV2 was not detected. They had the highest neutralizing antibody titres, appearing from 15 dpi. In these pigs a good cellular response was indicated by a peak in IFN-γ mRNA at 15 dpi. Pattern 2: five pigs contained low to moderate PCV2 titres at 15 dpi, remaining constant or decreasing towards 21 dpi. Lower neutralizing antibody titres were observed and no rise in IFN-γ was detected. Pattern 3: in one pig, a low PCV2-titre at 15 dpi dramatically increased towards 21 dpi. Although an antibody-response against PCV2 was mounted, no PCV2-neutralizing antibodies were detected. This pig also showed no rise in IFN-γ. The study indicates that variation in the onset of the adaptive immunity may account for variation in PCV2-replication between pigs. Absence of PCV2-neutralizing antibodies may be an important factor in the development of an increased virus replication.

Introduction

Porcine circovirus 2 (PCV2), a small single stranded DNA virus, is a member of the family of *circoviridae*. Amongst various other animal viruses, the *circoviridae* also contain some recently identified human viruses (Sagir *et al*., 2004). PCV2 is a widespread virus. In pig dense regions, it is known to infect all weaned pigs in conventional farms. In farms affected by the postweaning multisystemic wasting syndrome (PMWS), a disease that has been associated with PCV2-infection (Ellis *et al*., 1998; Morozov *et al*., 1998), the majority of PCV2 infections remain subclinical. In subclinically infected pigs, a humoral immune response is induced as shown by the production of antibodies (Labarque *et al*., 2000; Meerts *et al*., 2004) and the virus is efficiently cleared from the body. In some cases, in conditions that have not yet been elucidated, the infection of susceptible pigs will lead to the development of PMWS (Allan and Ellis, 2000). A characteristic feature of this disease is the presence of high amounts of PCV2 in lymphoid and non-lymphoid organs of affected pigs (Calsamiglia *et al*., 2002; Segalés and Domingo, 2002), while at the same time non-affected littermates are virologically negative or contain very low titres of PCV2 in their organs. The presence of high amounts of PCV2 in lymphoid organs of naturally PMWS-affected pigs is correlated with the presence of B and T lymphocyte depletion and monocyte infiltration (Quintana *et al*., 2001; Darwich *et al*., 2002), possibly clue factors in the pathogenesis of the disease.

During experimental inoculations of the virus, similar observations were made. When PCV2 was inoculated in susceptible pigs, high variation was observed in the level of PCV2-replication. In some pigs the virus was hardly detectable, whereas in others high titres of PCV2 were detected several weeks after inoculation (Allan *et al*., 2000a; Ladekjær-Mikkelsen *et al*., 2002; Rovira *et al*., 2002; Sanchez *et al*., 2003). Only in pigs with a high level of PCV2-replication, the typical histopathological lesions as described above were observed (Nielsen *et al*., 2003; Sanchez *et al*., 2004). When the clinical signs of PMWS could be reproduced in some of these studies, they were always found in pigs with a high level PCV2-replication (Ladekjær-Mikkelsen *et al*., 2002). The combined results of field and experimental studies suggest that pigs which experience high PCV2-replication are predisposed to develop PMWS, whereas pigs with low PCV2-replication are very unlikely to develop the disease. The elucidation of the mechanism that forms the basis of this event, could be an important

step in further understanding the pathogenesis of PCV2-infection and possibly of PMWS.

Since not all experimentally inoculated pigs with high PCV2-replication developed clinical symptoms, research has been focussed on identifying cofactors that influence the reproduction of the disease. Up till now, a synergy has been described between PCV2 and 2 other viruses: porcine reproductive and respiratory disease virus (PRRSV) (Allan *et al*., 2000a; Rovira *et al*., 2002) and porcine parvovirus (PPV) (Allan *et al*., 2000b). A general stimulation of the immune system has also been shown to influence the clinical outcome of the infection in gnotobiotic pigs (Krakowka *et al*., 2001). The mechanism of the synergistic effect between PCV2-infection on the one hand and other viruses or immune stimulation on the other hand, is not clarified. At present, the onset of the adaptive immune response against PCV2 and its influence on the kinetics of the PCV2-replication in the host have only partially been characterized. Starting from 10-14 days post inoculation (dpi) PCV2-specific antibodies (Ab) have been detected (Ladekjær-Mikkelsen *et al*., 2002; Sanchez *et al*., 2001). The appearance of the Ab coincided with a drop of PCV2 DNA copies in the blood of the host (Ladekjær-Mikkelsen *et al*., 2002; Pensaert *et al*., 2004). Neutralizing antibodies have only been described starting from 28 dpi (Pogranichnyy *et al*., 2000). The onset of the cellular immunity upon PCV2-infection and its influence on PCV2-replication, remain undocumented. Therefore, the aims of this study were to monitor the humoral and cellular immune responses upon PCV2-infection and to examine which immunological mechanism determines the development of the PCV2 replication kinetics in the pig and by this way possibly the clinical outcome of the infection.

Materials and methods

Pigs

Sixteen gnotobiotic piglets were derived from two Belgian landrace sows at 114 days of gestation by caesarean section. They were raised individually in a sterile environment in Horsfall-type isolators. At 19 days of age, they were randomly divided in 4 groups (group A: 4 pigs; group B: 8 pigs; group C: 2 pigs; group D: 2 pigs).

Virus inoculation

At 19 days of age, the pigs of group A (A1, A2, A3, A4) and group B (B1, B2, B3, B4, B5, B6, B7, B8) were oronasally and intraperitoneally inoculated with $10^{4.3}$ $TCID_{50}$ of PCV2 strain 1121 (Meehan *et al*., 2001). This PCV2 stock was propagated by four passages in PK-15 cells. The pigs of group C (C1, C2) and group D (D1, D2) were mock-inoculated with equal volumes of cell culture medium.

Cyclosporin treatment

Starting from 12 hours prior to the PCV2 or mock inoculation up to the end of the study, the pigs of groups A and C were treated with cyclosporin A (CysA) (Neoral-Sandimmun, Novartis Pharma, Vilvoorde) at a dose of 23 mg/kg/day (Matsuda and Kojasu, 2000) administered in 2 feedings per day, diluted in milk. By artificially suppressing the onset of the adaptive immune response upon the PCV2-infection with CysA, the influence of the adaptive immunity on the development of PCV2-infection was monitored.

Clinical monitoring and sampling

During the study, the pigs were monitored for clinical signs that have been described in association with PMWS: cachexia, depression, respiratory distress and jaundice (Allan and Ellis, 2000). At the end of the study, the pigs were weighed and the average weight of each group was calculated and compared as a marker for the general clinical condition of the pigs throughout the study.

In order to longitudinally follow the replication of PCV2 in the inguinal lymph nodes of the pigs, the lymph nodes were surgically sampled. At 10 days post inoculation (dpi) a biopsy was taken from the left inguinal lymph node and at 15 dpi a biopsy was taken from the right inguinal lymph node. In order to take lymph node biopsies, the pigs were anaesthetized by intramuscular injection of 2.2 mg tiletamine and 2.2 mg zolazepam (Zoletil®, Virbac) dissolved in 0.22 ml 2% Xylazine (XYL-M® 2%, VMD) per kilogram body weight. An incision was made in the overlaying skin and half the inguinal lymph node was excised. The wound was closed by mersilene sutures (Mersutures®, Ethicon). At 21 dpi, all pigs were euthanized and the remaining parts of both left and right inguinal lymph nodes were collected.

At 0, 10, 15 and 21 dpi, blood was taken (with 15 U/ml heparin) from all pigs in order to monitor the onset and evolution of both humoral and cellular immunity against PCV2.

All animal experiments performed in this study were approved by the ethical committee of the Faculty of Veterinary Science, Ghent University, Belgium.

Longitudinal monitoring of PCV2-replication in inguinal lymph nodes

Since it has been shown that the PCV2 titre in the inguinal lymph nodes is very well correlated with the titre in other lymphoid and non-lymphoid target organs (Ladekjær-Mikkelsen *et al*., 2002; Sanchez *et a*l., 2003), the titre in the inguinal lymph node can be considered to be a good marker for the general replication of the virus in the body of the host. At 10, 15 and 21 dpi, PCV2 titres were determined in the biopsies and inguinal lymph nodes of all pigs. Ten % tissue suspensions were made of a part of the biopsies and lymph nodes and the PCV2 titre was determined by virus titration on PK-15 cells as described elsewhere (Sanchez *et al*., 2001). The detection limit of this assay was $10^{2.4}$ $TCID_{50}$ PCV2/gram lymph node tissue.

Monitoring of the adaptive immune response

Peripheral blood mononuclear cells (PBMCs) were separated from blood, collected at different time points, by density centrifugation at 750 x g on Ficoll-Paque® (Amersham Pharmacia Biotech AB, Upsalla, Sweden) in order to monitor the cellular immune response. Plasma was collected to determine the humoral immune response against PCV2.

1. Humoral immune response: The presence of PCV2-specific antibodies in plasma was determined by an immunoperoxidase monolayer assay (IPMA) as described earlier (Sanchez *et al*., 2001). This assay detects all antibodies that react with PCV2 antigens and does not discriminate between neutralizing or non-neutralizing antibodies. This discrimination was made by using a seroneutralization assay adapted from the one described by Delputte *et al*. (2004). Briefly, $10^{3.7}$ $TCID_{50}$ PCV2 was incubated for 1 hour with 25% of plasma at 37°C. Subsequently this mixture of virus and plasma was inoculated on PCV-negative PK-15 cells. After 1 hour of incubation, the inoculum was removed, cell cultures were washed twice and

new medium was added. At 36 hours post inoculation, after the first replication cycle of PCV2 was completely finished (Meerts *et al*., 2004), the cultures were fixed and PCV2-infected PK-15 cells were stained by an immunoperoxidase reaction using polyclonal anti-PCV2 antibodies. The number of infected cells was counted by light microscopy. The PCV2-neutralizing capacity of the plasma was determined by calculating the reduction in PCV2-infected cells when compared to cells inoculated with PCV2 incubated with plasma of the same pig from day 0 (before PCV2-inoculation).

2. IFN-γ response: In this study, the level of IFN-γ mRNA in freshly isolated PBMCs was determined by real-time reverse transcriptase PCR as described by Verfaillie *et al.* (2001). Briefly, RNA from PBMCs was extracted with the Trizol® reagent (invitrogen, Carlsbad, USA) according to the manufacturer's instructions. In a next step, RNA was transformed into cDNA by means of reverse transcriptase (Superscript™ II reverse transcriptase, invitrogen, Carlsbad, USA). In a final step, the number of cDNA copies coding for IFN-γ and cyclophilin was determined by REAL-time PCR using the Lightcycler FastStart DNA Master SYBR Green I kit (Roche applied science, Penzberg, Germany) in a Roche LightCycler (Roche applied science, Penzberg, Germany). The level of IFN-γ mRNA was compared with the level of mRNA transcription of the household gene cyclophilin that served as an internal control. The level of IFN-γ mRNA was determined by calculating the ratio of IFN-γ mRNA to cyclophilin mRNA. To circumvent individual variation between pigs, the results of this test were expressed as the IFN-γ mRNA expression level at the day of testing, divided by the level at day 0.

Statistical analysis

The statistical significance of differences between medians of different groups was tested by applying two-sided Mann-Whitney tests. Bonferroni corrections for multiple comparisons at different time points were used. Differences were considered significant when $p < 0.05$.

Results

Clinical monitoring of pigs

During the study, no clinical signs were observed in pigs of group B (non-CysA-treated/PCV2-inoculated) and group D (non-CysA-treated/non-PCV2-inoculated). All pigs in groups A and C (respectively CysA-treated/PCV2-inoculated and CysA-treated/non-PCV2-inoculated) showed diarrhoea starting between the fifth and the tenth day of CysA treatment, lasting up to the end of the study. At the end of the study these pigs showed a moderate jaundice. Other clinical signs such as respiratory distress, anorexia or depression were not observed during the study. The excision of lymph node biopsies did not cause noticeable discomfort to the pigs. At the end of the study, no major differences were observed between the mean body weight of pigs of different groups (group A: 5.1 kg; group B: 4.9 kg, group C: 5.3 kg; group D: 5.4 kg) or between body weights of individual pigs in the same group.

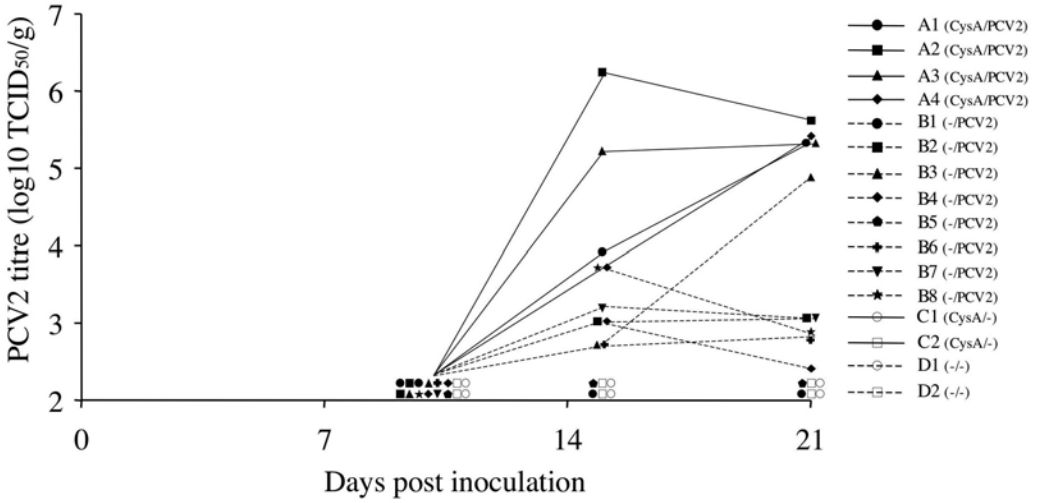
Longitudinal monitoring of PCV2-replication in inguinal lymph nodes

The results of the evolution of PCV2 replication in inguinal lymph nodes of the pigs are shown in Figure 1. In the inguinal lymph nodes of non-PCV2-inoculated pigs (groups C and D), PCV2 was never detected. At 10 dpi, PCV2 could not be detected in the inguinal lymph nodes of any of the PCV2-inoculated pigs. Starting from 15 dpi, PCV2 was detected in inguinal lymph nodes of 10 out of 12 PCV2-inoculated pigs and different evolutions in PCV2-titres over time were observed. The pigs from group A (CysA-treated/PCV2-inoculated) showed significantly higher median titres at 15 and 21 dpi compared to the pigs from group B. Two of these pigs (A2 and A3), the pigs with the highest titre at 15 dpi (respectively $10^{6.2}$ and $10^{5.2}$ $TCID_{50}$/gram lymph node tissue), reached the maximal level of PCV2 replication at this time point, the two other pigs in this group (A1 and A4) showed an increase in PCV2-titre from 15 dpi ($10^{3.7}$ $TCID_{50}$/gram lymph node tissue) to 21 dpi ($10^{5.2}$ and $10^{5.4}$ $TCID_{50}$/gram lymph node tissue).

In group B (non-CysA-treated/PCV2-inoculated), three different patterns in PCV2-replication were observed. From the lymph nodes of two out of eight pigs in this group (B1 and B5), PCV2 could never be isolated during the study (pattern 1).

PCV2 could be isolated from the inguinal lymph nodes of the six other pigs in this group at various amounts. Five of these pigs (B2, B4, B6, B7, B8) showed a maximal PCV2-titre at 15 dpi (between $10^{2.7}$ and $10^{3.7}$ $TCID_{50}$/gram lymph node tissue), after which the titre remained similar or decreased towards 21 dpi (pattern 2). In one pig (B3) a very distinct evolution in PCV2 replication was observed. At 15 dpi this pig showed one of the lowest PCV2-titres of group B ($10^{2.7}$ $TCID_{50}$/gram lymph node tissue). A dramatic increase in PCV2-replication was observed towards 21 dpi ($10^{4.9}$ $TCID_{50}$/gram lymph node tissue) (pattern 3). The evolution in PCV2-replication in this pig was comparable with the evolution in pigs A1 and A4 (CysA-treated/PCV2-inoculated) in which also a steep increase in the PCV2-titre was observed between 15 and 21 dpi. At 21 dpi, the PCV2-titre in the inguinal lymph nodes of this pig was between 1.6 and 2.2 log10 times higher compared to the titres in the other pigs of group B and was situated closer to the mean of the titres of the artificially immunosuppressed pigs of group A than it was to the mean of group B.

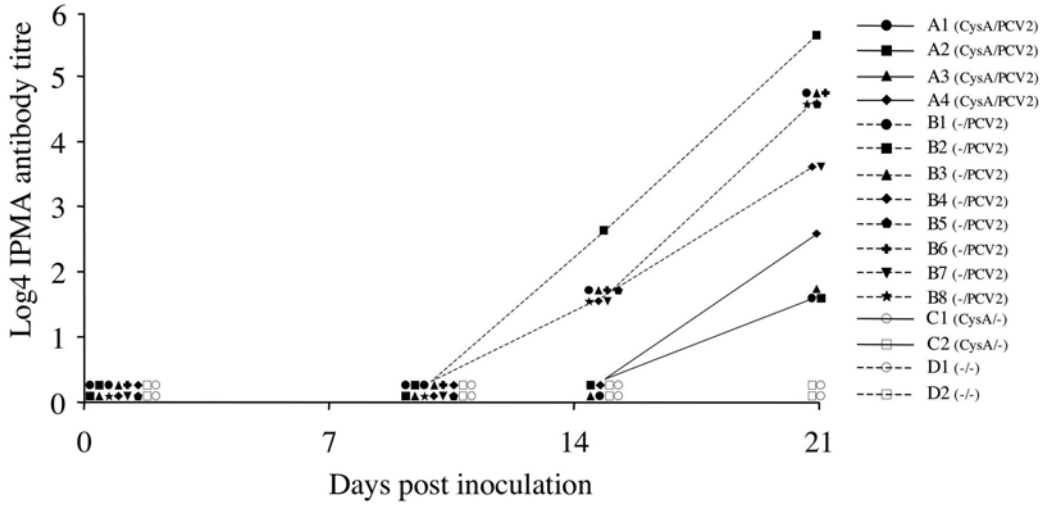
Figure 1. Evolution of PCV2 titres in inguinal lymph nodes of PCV2-inoculated and non-PCV2-inoculated pigs. The PCV2-titres at 10 and 15 dpi are titres in the lymph node biopsies, excised at that day. The titres at 21 dpi are the average titres of the remaining parts of left and right inguinal lymph nodes collected at euthanasia.



Monitoring of the PCV2-specific adaptive immune response

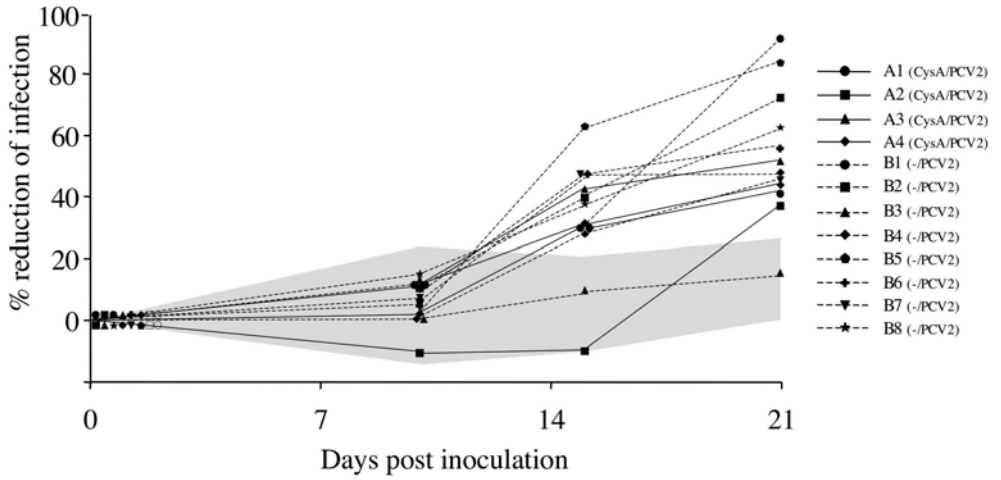
1. Humoral immune response: The IPMA Ab titres against PCV2, as determined in the plasma of pigs at different time points during the study, are shown in Figure 2. The non-PCV2-inoculated pigs of groups C and D did not show PCV2-specific antibodies during the study. This confirms that they were not infected with PCV2. All PCV2-inoculated pigs showed seroconversion against PCV2 during the study. In all pigs of group B (non-CysA-treated/PCV2-inoculated), anti-PCV2 Ab were detected for the first time at 15 dpi (IPMA titres ranging from $4^{1.7}$ to $4^{2.7}$), clearly showing that all pigs in group B got infected upon PCV2-inoculation. The IPMA Ab titre increased in these pigs towards 21 dpi (IPMA titres ranging from $4^{3.7}$ to $4^{5.7}$). The pigs of group A (CysA-treated/PCV2-inoculated) also seroconverted against PCV2 but Ab were detected for the first time at 21 dpi. At this time point the Ab titres in these pigs were similar to the Ab titres in pigs of group B at 15 dpi (IPMA titres ranging from $4^{1.7}$ to $4^{2.7}$).

Figure 2. Evolution of anti-PCV2 antibodies (IPMA) in plasma of PCV2-inoculated and non-PCV2-inoculated pigs



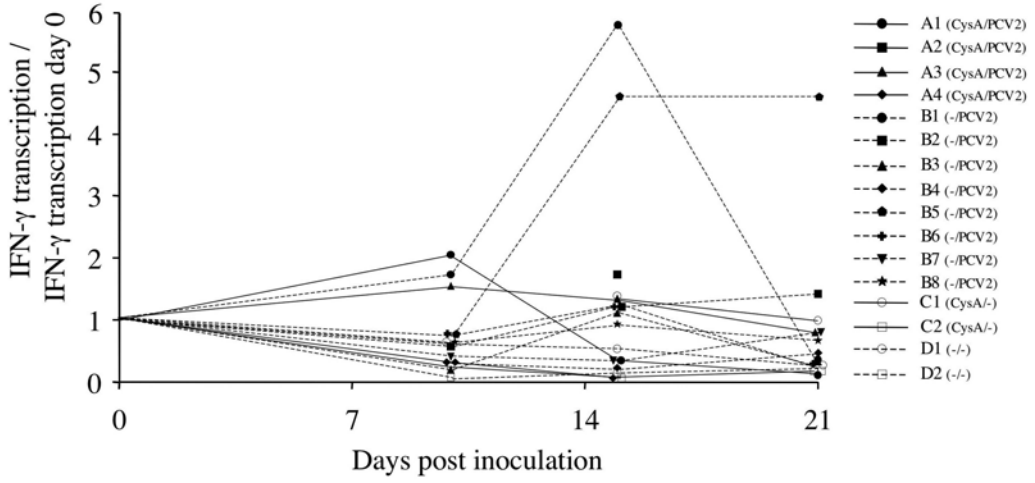
The evolution of PCV2-neutralizing Ab (neutralization of PCV2-infection) in plasma is shown in Figure 3. Pigs of groups C and D (non-PCV2-inoculated pigs) did not contain neutralizing Ab in their plasma during the study. At 10 dpi, the median outcome of the PCV2-inoculated pigs did not differ significantly from the median of the non-PCV2-inoculated pigs, indicating that at this time no evidence for the presence of PCV2-neutralizing Ab in the plasma of PCV2-inoculated pigs was found. At 15 and 21 dpi a significant difference was found between the median outcome of non-PCV2-inoculated and PCV2-inoculated pigs indicating the presence of PCV2-neuralization in the sera of PCV2-inoculated pigs. Starting from 15 dpi, PCV2-neutralizing Ab were detected in 10 out of 12 PCV2-inoculated pigs (all except A2 and B3). The PCV2-neutralization at this time point ranged from 30% to 62%, increasing towards 21 dpi (ranging from 41% to 93%). At 21 dpi, neutralization was also found in plasma of pig A2 (neutralization of 38%). In the plasma of one pig of group B, pig B3 (the pig with the strong increase in PCV2-replication between 15 and 21 dpi), PCV2 neutralization could not be detected during the course of the study. The highest PCV2 neutralization at 21 dpi was found in plasma of pigs B1 and B5, the pigs from which no PCV2 could be isolated during the study.

Figure 3. PCV2-neutralizing activity in plasma of PCV2-inoculated and non-PCV2-inoculated pigs. The lines represent values of individual PCV2-inoculated pigs. The grey area represents the range (minimal and maximal value) in which the values of the four non-PCV2-inoculated pigs were situated



2. IFN-γ response: In all pigs, a basal level of IFN-γ gene transcription in PBMCs was detected prior to PCV2-inoculation. The evolution of IFN-γ mRNA expression levels in PBMCs of individual pigs during the study is shown in Fig. 4. Due to poor RNA-quality after isolation, the results of some pigs at some time points were not available (C1: 10 dpi; A2: 10 and 21 dpi; A4: 21 dpi). Non-PCV2-inoculated pigs (groups C and D) generally showed low IFN-γ mRNA expression levels (between 0.02 and 1). All pigs from group A (CysA-treated/PCV2-inoculated) and six out of eight pigs from group B (non-CysA-treated/PCV2-inoculated) showed IFN-γ mRNA expression levels ranging in-between 0.1 and 2.3. In these pigs, no clear peak in IFN-γ mRNA expression levels in PBMCs was observed during the study. Two pigs from group B (B1 and B5) showed a peak in IFN-γ mRNA at 15 dpi (respectively to 5.8 and 4.6), decreasing (B1) or remaining at the same level (B5) towards 21 dpi. These pigs were the two inoculated pigs from which no PCV2 could be isolated during the study.

Figure 4. IFN-γ mRNA expression levels in PBMCs of PCV2-inoculated and non-PCV2-inoculated pigs



Discussion

In the present study, an effect of the PCV2-adaptive immune response on the replication of the virus was observed. A detectable humoral and cellular immune response was observed in some pigs and was correlated with a replication level of the virus below the detection limit of the used assay. On the other hand, a correlation was described between a complete absence of PCV2-neutralizing antibodies and the presence of an increased PCV2-replication in one pig. This study was performed in gnotobiotic piglets that did not encounter any immunologic stimulation before PCV2-infection and therefore their immune response might deviate to some extent from pigs raised in a more conventional environment (Mehrazar *et al*., 1993).

In this study, PCV2-replication in lymphoid organs was for the first time followed by using a method of inguinal lymph node biopsies. This way, titres could be obtained at different time points in the same pig and PCV2-replication kinetics could be compared between individual pigs. Through this technique, the number of pigs, necessary to demonstrate significant differences, could be reduced. At 10 dpi, a discrepancy was detected between the experimental inoculation performed in the present study and similar inoculations performed previously in our laboratory (Sanchez *et al*., 2003). In previous experimentally inoculated piglets, PCV2-titres could already be detected starting from 10 dpi. At this time, the titres in the internal organs of the piglets (including the inguinal lymph nodes) were relatively high. In the present study, PCV2 could not be detected in the inguinal lymph nodes of the inoculated piglets at 10 dpi despite identical procedures. The only differences between these pigs were their age at inoculation (1 days of age versus 19 days of age in this study) or minor environmental factors that may have occurred without our knowledge. It shows that minor factors can have important consequences on the further evolution of PCV2-replication in its host. This possibly explains the high variation in virological and clinical results from different PCV2-inoculation experiments performed by different research groups (Ladekjær-Mikkelsen *et al*., 2002, Sanchez *et al*., 2003).

Although the eight PCV2-inoculated/non-CysA-treated pigs used in this study, were derived from only two litters, three distinct patterns in PCV2-replication were observed. Pattern 1: from the lymph nodes of two out of these eight pigs (25%) no PCV2 could be isolated. These pigs did get infected by the virus as shown by their

normal IPMA antibody response. Pattern 2: five pigs (63%), the majority of the pigs, showed a low to moderate PCV2-replication remaining constant or decreasing towards the end of the study. Pattern 3: a very different evolution was observed in the last pig (1/8 or 13%) which suffered an intense increase in PCV2-replication from 15 to 21 dpi. To fully understand the pathogenesis of a PCV2-infection in its host, it is important to understand the basis for these different patterns.

An important mechanism contributing to the variation in virus replication seems to be represented by the onset and efficacy of the adaptive immune response of the individual pig against PCV2-infection. This could be concluded from correlating the PCV2-replication patterns with the immunologic parameters investigated in this study. During the study, four PCV2-inoculated pigs were treated with CysA to artificially suppress their adaptive immunity (group A). CysA is known to interrupt the T-cell receptor signal transduction pathway and by doing this, inhibiting T-cell proliferation (Ho *et al*., 1996; Matsuda and Koyasu, 2000). By this mechanism, CysA treatment inhibits the onset of the cellular immune response and the thymus-dependent humoral immune response upon virus-infection (Charan *et al*., 1986). CysA treatment has already been shown to render pigs more susceptible to PCV2-replication (Krakowka *et al*., 2002). The CysA-treated pigs showed a delay in production of IPMA-antibodies against PCV2 and low levels of neutralizing antibodies compared to the non-CysA-treated pigs. The late onset of antibody production indicates that the CysA treatment inhibited the humoral immunity but did not completely block it. Since IFN-γ is produced by activated Th1-cells, its transcription in a virus-inoculated host can be used to monitor the onset of the cellular immunity against infection (Johnsen *et al*., 2002). No rise in IFN-γ mRNA expression was detected in the CysA-treated pigs. At 21 dpi, all four of the CysA-treated piglets contained PCV2-titres which were significantly higher compared to the titres in seven out of eight non-CysA-treated, PCV2-inoculated pigs at the same time point, indicating that the adaptive immunity plays an important role in controlling PCV2-infection. When the evolution in PCV2-replication in CysA-treated pigs and in non-CysA-treated pigs is compared, it is clear that the pigs with replication pattern 1 and 2 mounted an adaptive immune response upon PCV2-infection that was able to control the level of PCV2 replication. The PCV2-titre at 21 dpi of the non-CysA-treated pig with pattern 3, did not significantly differ from the titres of the CysA-treated pigs,

indicating that the virus was able to replicate as intensively in this pig as in the artificially immunosuppressed pigs. Pigs with pattern 1 mounted the most efficient adaptive immune response against the virus. This was shown by the peak in IFN-γ mRNA in PBMCs, indicating a Th1-mediated immune response and by their very high PCV2-neutralizing antibody levels. The efficient immunological response in these pigs coincided with a strong protection against the virus. In contrast, the pig showing pattern 3 did not show a peak in IFN-γ mRNA, neither did it produce any PCV2-neutralizing antibodies in its plasma. This coincided with a weak virological protection. In between these opposites, the pigs with pattern 2 did not show an IFN-γ mRNA peak but did have PCV2-neutralizing antibodies. Differences in IFN-γ mRNA expression levels were most likely not induced by other infectious agents. Bacteriological examinations of internal organs and faeces demonstrated that the pigs were bacteriologically negative and serologic examinations confirmed that the pigs remained seronegative to various common porcine viruses throughout the study (data not shown). From our study it cannot be concluded whether the pigs in which an increase in IFN-γ mRNA expression level in PBMCs was not detected, mounted no cellular immune response at all. It is possible that an increase in IFN-γ mRNA could have been present in lymphocytes present in lymph nodes or other target organs. This was not investigated in the present study.

The present study is the second study describing the existence of PCV2-neutralizing antibodies. In contrast to the previous study (Pogranichnyy *et al*., 2000), in which the neutralizing antibodies were detected starting from 28 dpi, neutralizing antibodies could be detected starting from 15 dpi, simultaneously with the onset of the total anti-PCV2 antibody production. The difference between these results is probably based on a difference in sensitivity of the applied techniques. The total absence of neutralizing antibodies in the pig with pattern 3, while at the same time other pigs had relatively high levels, was a remarkable finding since this pig was able to produce IPMA PCV2 antibody titres similar to its group mates. A possible explanation for the absence of neutralizing antibodies is a difference in epitope-specific responses as has been shown for other viruses (Green *et al.*, 1990; Jacobs and Kimman 1994; Lambkin and Dommock, 1995). The inability of a subpopulation of pigs to raise neutralizing antibodies against PCV2 may have an important effect on the further evolution of the infection and perhaps on its clinical outcome. It indicates that high titres of IPMA

anti-PCV2 antibodies do not always represent a good protective immunity of the host, a feature that will have to be considered when vaccines against PCV2 are optimised.

In the present study, all CysA-treated pigs developed diarrhoea and jaundice during the study. However, these clinical signs were observed in both PCV2-inoculated and PCV2-non-inoculated pigs, indicating that they were not caused by the infection. The presence of an increased and prolonged PCV2-replication in one pig did not induce clinical symptoms as described in PMWS-affected pigs during the time of the study. When PMWS was reproduced in PCV2-inculated animals, the first clinical signs always appeared within the first two or three weeks (Harms *et al*., 2001; Ladekjær-Mikkelsen *et al*., 2002). The absence of clinical signs in the present study suggests that an additional factor is needed for the reproduction of disease. When it is considered that a high PCV2-replication is crucial in the pathogenesis of PMWS, as sufficiently shown in literature, it can be assumed that pigs in field conditions which are not able to mount neutralizing antibodies, will have a higher chance to suffer the consequences of the infection compared to pigs that are able to efficiently clear the virus. In order to prevent PCV2-related clinical signs and their associated economic losses, two different strategies can be followed. The first strategy implies the identification and elimination of the second factor responsible for the induction of disease upon high PCV2-replication. This might be a complicated matter, especially when this factor would be an enzootic virus as has been suggested (Rovira *et al*., 2002; Allan *et al*., 2000b) or even a general stimulation of the immunity (Krakowka *et al*., 2001). The second strategy involves the ability of the pig to control the PCV2-replication. In the present study, a gradation was observed in the ability of pigs to mount a good adaptive immune response against PCV2-infection. The pig with pattern 3 (high PCV2-titres) was clearly more susceptible to PCV2-replication than the two pigs showing pattern 1 (no PCV2 detected) with the majority of the pigs (five showing pattern 2) caught in between. The differences in susceptibility to PCV2-infection and sustained replication were in this study correlated with the adaptive immune response mounted in the pigs. The results indicate that on long term, the selection of pigs towards pattern 1 may be the best way to avoid PCV2-related problems.

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5.2.

Absence of porcine circovirus type 2 neutralizing antibodies in pigs with postweaning multisystemic wasting syndrome

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Summary

In a previous study, it was demonstrated that high replication of Porcine circovirus 2 (PCV2) in one gnotobiotic pig was correlated with the absence of PCV2-neutralizing antibodies. In the present study, it was shown that this correlation could also be found in SPF pigs in which PMWS was experimentally reproduced and in naturally PMWS-affected pigs. When looking at the total anti-PCV2 antibody titres, PMWS-affected and healthy animals seroconverted at the same time point and titres in PMWS-affected animals were only slightly lower compared to those in healthy animals. In healthy animals, the evolution of PCV2-neutralizing antibodies coincided with that of total antibodies. In PMWS-affected animals, neutralizing antibodies could not be found (field studies) or were detected in low titres between 7 and 14 dpi (experimentally inoculated SPF pigs). Differences were also found in the evolution of specific antibody isotype titres against PCV2. In healthy pigs, IgM antibodies persisted until the end of the study whereas in PMWS-affected pigs they quickly decreased or remained at low titres. The mean titres of other antibody isotypes (IgG1, IgG2 and IgA), were slightly lower in PMWS-affected pigs compared to their healthy group mates at the end of each study. This study describes important differences in the development of the humoral immune response between pigs that get subclinically infected with PCV2 and pigs that experience a high level of PCV2-replication which in 3 of 4 experiments led to the development of PMWS. These observations may contribute to a better knowledge of the pathogenesis of a PCV2-infection.

Introduction

Porcine circovirus type 2 is a member of the *Circoviridae* family. This family contains small viruses with a circular single stranded DNA genome, including a number of avian and mammal viruses that are known to cause persistent infections in their host. Porcine circovirus 2 (PCV2) and chicken anemia virus are able to maintain a high level of replication up to one month or longer after experimental inoculation (Bolin *et al*., 2001; Imai *et al*., 1999). The human hepatitis-related TT-virus, also a member of the *circoviridae* family (Nishizawa *et al*., 1997), is able to maintain a constant viremia for a period of more than 3 years in infected patients (Lefrère *et al*., 2000). The ability of these small viruses to persist and replicate for such a long time in the infected host, indicates a failure of the host's immunologic response to efficiently recognize and/or remove virus particles or virus-infected cells from the body.

The economical importance of PCV2 lies in its association with the postweaning multisystemic wasting syndrome (PMWS) (Ellis *et al*., 1998), a condition in weaned pigs observed for the first time in 1991 (Harding *et al*., 1996). The syndrome is characterized by clinical signs such as anaemia, jaundice and severe weight loss and histopathological signs including lymphocyte depletion and infiltration of monocytes. It is generally accepted that secondary factors including other viral infections such as porcine parvovirus (Allan *et al*., 2000a) or porcine reproductive and respiratory syndrome virus (Allan *et al*., 2000b) or stimulations of the immune system (Krakowka *et al*., 2001), influence the clinical outcome of the infection. A high and persisting PCV2-replication level was found to form a crucial factor in the pathogenesis of the disease. The demonstration of an intense PCV2-replication was therefore incorporated in the case definition of PMWS in order to differentiate the disease from other chronical disorders leading to the manifestation of similar clinical symptoms (Segalés and Domingo, 2002).

After experimental PCV2-inoculation, a high variation in virus replication level and duration has been described between pigs (Ladekjær-Mikkelsen *et al*., 2002; Sanchez *et al*., 2003). In a previous study, the evolution in PCV2-replication was monitored in individual gnotobiotic pigs (Meerts *et al*., 2005). During the first two weeks of the experiment, the evolution of the PCV2-titre in inguinal lymph nodes of inoculated pigs showed little variation. At later stages of the infection, two patterns

developed. In most pigs, PCV2-titres decreased between 15 and 21 dpi. One of the PCV2-inoculated pigs, showed a dramatic increase in PCV2-replication in this period, resulting in a significantly higher level of PCV2-replication at 21 dpi. Similar observations were made in other studies (Ladekjær-Mikkelsen *et al*., 2002; Sanchez *et al*., 2003). From these observations, it could be concluded that not all pigs are equally able to deal with a PCV2-infection. When the relation between the onset of the adaptive immune response against PCV2 was monitored in these pigs, an interesting observation was made. Using a very sensitive PCV2-neutralization assay, PCV2-neutralizing antibodies could be detected in all PCV2-inoculated pigs starting from 15 dpi, except in the pig with high PCV2-replication. This study suggested a correlation between the absence or delayed production of PCV2-neutralizing antibodies and an increased level of replication of the virus in experimentally inoculated pigs. The latter study was performed in gnotobiotic piglets. Previously, it has been shown that the adaptive immune response against viral infections in gnotobiotic pigs which are raised in a sterile environment may deviate to some extent from the response of pigs in a conventional environment (Mehrazar *et al*., 1993; Butler *et al*., 2002; Souza *et al*., 2004). Therefore, the observation made in PCV2-inoculated gnotobiotic piglets should be confirmed in naturally infected and experimentally inoculated conventional pigs.

The aim of the present study was to investigate whether the lack of PCV2-neutralizing antibodies could also be detected in natural or experimentally reproduced PMWS-affected pigs and to characterize more in detail the humoral immune response against PCV2 in PMWS-affected and healthy animals.

Materials and methods

Sera

An overview of the sera used in the present study, is shown in Table 1. Sera originating from four different studies were included. A first set of sera were derived from two experimental PCV2-inoculation studies, one performed in 19-day-old gnotobiotic pigs and another performed in 21-day-old PCV2-negative conventional specific pathogen free (SPF) pigs. A second set of sera originated from two field studies performed in Belgium and Denmark.

1. Experimentally inoculated gnotobiotic pigs (Meerts *et al*., 2005): 8 gnotobiotic piglets were inoculated with PCV2 ($10^{4.3}$ $TCID_{50}$ of strain 1121 intraperitoneally and oronasally) at 19 days of age. Blood was taken from these piglets at 0, 10, 15 and 21 days post inoculation (dpi). In this experiment, PMWS was not observed but one pig showed a high level of PCV2-replication at the end of the experiment ($10^{4.3}$ $TCID_{50}$/g inguinal lymph node) compared to the other PCV2-inoculated pigs (average of $10^{2.3}$ $TCID_{50}$/g inguinal lymph node). This pig produced no PCV2-neutralizing antibodies.

2. Experimentally inoculated SPF pigs (Ladekjær-Mikkelsen *et al*., 2002): 10 PCV2-negative SPF-pigs were inoculated with PCV2 ($10^{6.3}$ $TCID_{50}$ of strain OSU3 intranasally) at the age of 3 weeks. Blood was taken at 0, 3, 7, 10, 14 and 21 dpi. Four out of 10 inoculated pigs developed PMWS and died before the end of the study (2 pigs at 14 dpi, 2 pigs at 21 dpi). These pigs showed high titres of PCV2 at the time they died (average of $10^{5.1}$ $TCID_{50}$/g inguinal and prescapular lymph node). At the end of the study, no PCV2 could be isolated from lymph nodes of pigs that did not develop PMWS.

3. Belgian field study (Meerts *et al*., 2004): during this study, blood was taken every two weeks from 9 piglets originating from 2 litters on PMWS-affected farms in Belgium. This serological follow-up started before the appearance of PMWS-related clinical signs (4 weeks of age) and ended at 14 weeks of age, the age after which new cases of PMWS seldom occur. In each litter, one piglet developed PMWS during the study and died before the end of the study.

4. Danish field study (Kristensen *et al*., 2005): in this field study, pigs originating from PMWS-affected and healthy farms were weaned and brought to the same unit. Blood was taken at the day the pigs were mingled (day 0) and 3 and 6 weeks later.

During the study, a number of pigs developed PMWS and some of these affected pigs died before the end of the study. In the present study, sera were included originating from 13 subclinically infected pigs (healthy) and 13 pigs that developed PMWS during the follow-up. Eight affected pigs died before the last blood sampling occurred, five pigs developed PMWS but survived until the end of the study.

Table 1. Sera used in the present study. high*: $\geq 10^{4.3}$ $TCID_{50}$/gram lymph node: NA**: not available since PCV2-titres were not obtained from healthy pigs. All PMWS-affected pigs did contain high titres of PCV2

Origin of pigs	PCV2-infection	Serum samples available	Number of pigs with	
			high* PCV2-replication	clinical signs of PMWS
Gnotobiotic	experimental	0, 10, 15, 21 dpi	1/8	0/8
SPF conventional	experimental	0, 7, 10, 14, 21 dpi	4/10	4/10
Field cases (Belgium)	natural	4, 6, 8, 10, 12, 14 weeks of age	NA**	2/10
Field cases (Denmark)	natural	0, 3, 6, 9 weeks after mingling	NA**	13/26

Immunoperoxidase monolayer assay (IPMA)

The total titre of anti-PCV2 antibodies (IPMA-Ab) was determined using the IPMA as described previously by Labarque *et al*. (2000a). This assay does not discriminate between neutralizing and non-neutralizing antibodies nor does it detect specific antibody isotypes.

Isotype-specific IPMA

The individual levels of antibody isotypes IgG1, IgG2, IgA and IgM against PCV2 in sera were determined by an isotype specific IPMA. PCV2-infected PK-15 cell cultures were fixed and incubated with 4-fold dilution series of the tested sera. After 90 minutes of incubation, the cell cultures were washed thoroughly (4 times in phosphate buffered saline + 0,1% Tween 80) to remove unbound antibodies. Afterwards, monoclonal antibodies directed against porcine IgM, IgA, IgG1 or IgG2

were added and incubated for 60 minutes (Labarque *et al*., 2000b). After additional washing, peroxidase-conjugated goat polyclonal antibodies against mouse immunoglobulins were added (Goat-anti-Mouse-HRP, DAKO) and peroxidase activity was visualized by adding 3-amino-5-ethylcarbazole.

Classical seroneutralization assay (Classical SN)

With this assay, PCV2-neutralizing antibodies were detected using the classical protocol as has been described for many other viruses (Nauwynck *et al*., 1999; Van Reeth *et al*., 2003). Two-fold dilution series of the tested serum were prepared in MEM. The lowest dilution used in the test was 50%. Twenty-five µl of these serial diluted sera were incubated for 1 hour at 37°C with a fixed number of infectious particle of PCV2 ($10^{3.7}$ $TCID_{50}$ of strain Stoon-1010 in 25 µl). This mixture was afterwards added to 50% confluent PK-15 cells in a 96-well plate. After 1 hour of incubation at 37°C, the inoculum was removed, cell cultures were washed twice with MEM and culture medium was added. Inoculated cultures were further incubated at 37°C in an atmosphere containing 5% CO_2. After 36 hours of incubation, the cells were fixed and stained with the immunoperoxidase assay as described before. By light microscopy, it was observed at which concentration of the serum, PCV2-positive cells could still be seen. The neutralization titre with this assay was calculated as the reciprocal of the highest dilution of the serum that was able to completely block PCV2-infection in PK-15 cells. Since the lowest serum dilution contained 25% serum, the detection limit of this assay was 2.

Sensitive seroneutralization assay (sensitive SN)

It has previously been shown that PCV2-neutralizing antibody titres are only slowly induced (Pogranychnyy *et al*., 2000). Therefore, a more sensitive technique was developed. When no PCV2-neutralizing antibodies could be detected with the Classical SN, PCV2-neutralizing antibodies were detected by using the more sensitive seroneutralization described by Meerts *et al.* (2005). A similar neutralization assay had previously also been used to detect neutralizing antibodies against porcine reproductive and respiratory syndrome virus (Delputte *et al.*, 2005). Briefly, a standard infectious dose of PCV2 ($10^{3.7}$ $TCID_{50}$ of strain Stoon-1010) was incubated

for 1 hour at 37°C with 25% of the serum. Afterwards, this virus-serum mixture was inoculated on PCV-negative PK-15 cells. After 1 hour, the inoculum was removed, the cell cultures were washed 2 times, new cell medium was added and cultures were maintained at 37°C in the presence of 5% CO2. After 36 hour of incubation, when the first replication cycle of PCV2 in PK-15 cells is completed, the cells were fixed and stained for PCV2 antigens using an immunoperoxidase technique. The number of PCV2-infected cells was counted and compared to the number of infected cells in a PK-15 culture inoculated with a PCV2 stock treated with the serum of the pig before inoculation. The result of this seroneutralization assay is expressed as a percentage of reduction of PCV2-infection.

Statistical analysis

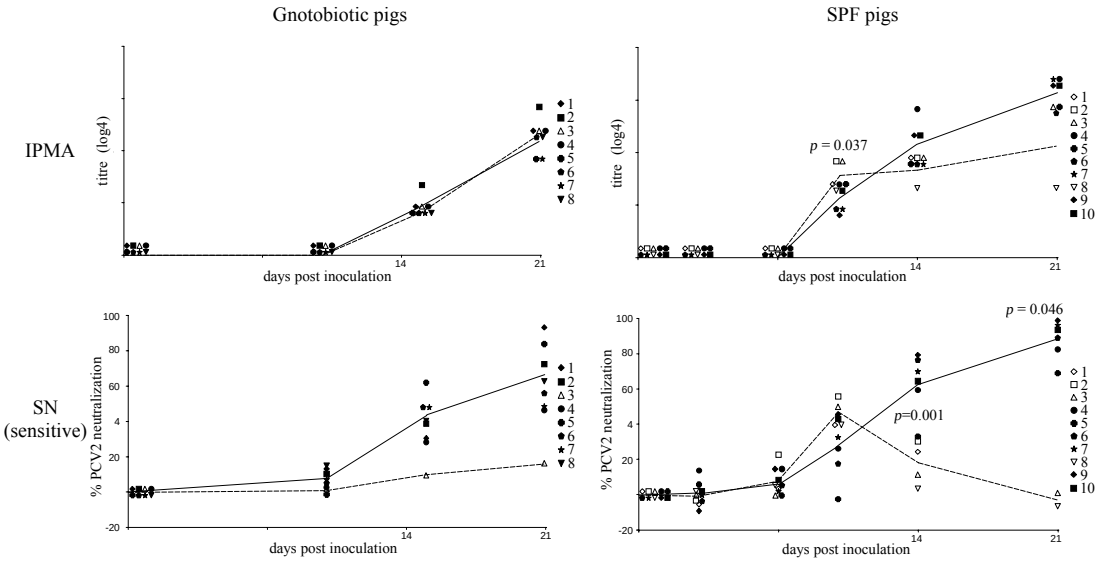
Statistical significance of differences between mean antibody titres of groups of subclinically infected pigs or pigs that developed PMWS, was calculated using the Kruskal-Wallis test. Differences were considered significant when $p < 0.05$.

Results

Total PCV2-specific and PCV2-neutralizing antibody titres in experimentally inoculated gnotobiotic and conventional SPF pigs

The total anti-PCV2 antibody profiles in experimentally inoculated gnotobiotic and conventional SPF pigs are shown in Figure 1. The first anti-PCV2 antibodies were detected at 10 dpi in SPF pigs. At that time PMWS-affected SPF pigs showed significantly higher IPMA titres compared to their healthy group mates ($p = 0.037$). In gnotobiotic pigs no antibodies were detected before 15 dpi. Antibody titres increased in a time-dependent manner until the end of the study or until the pig died. Only in one PMWS-affected SPF pig (pig 3), a plateau was reached starting from 10 dpi. At 21 dpi, the antibody titre in this pig was low (2.7 log4) when compared to the other pigs (5.7 – 6.7 log4).

Figure 1. Course if IPMA and SN (sensitive) antibody titres in experimentally PCV2-inoculated gnotobiotic and conventional SPF pigs. Full symbols: pigs with low PCV2 replication (gnotobiotic) or subclinically infected pigs (SPF); empty symbol: pig with high PCV2-replication (gnotobiotic) or PMWS-affected pigs (SPF). Full line: mean antibody titre of the pigs with low PCV2 replication (gnotobiotic) or healthy pigs (SPF). Dashed line: antibody titre of pig with high PCV2-replication (gnotobiotic) or mean antibody titre of PMWS-affected pigs (SPF). When the difference between both means was significant, the *p*-value was shown.



With the Classical SN, no PCV2-neutralizing antibodies were detected in any pig until the end of the studies at 21 dpi. Therefore, the sensitive SN was applied in these studies. With this assay, PCV2-neutralizing antibodies were detected in gnotobiotic and SPF pigs starting from the same time point when anti-PCV2 antibodies were detected with the IPMA (10 dpi in SPF and 15 dpi in gnotobiotic pigs). In seven gnotobiotic pigs (pigs with low PCV2-replication) and in six SPF pigs (pigs that did not develop PMWS) the evolution in PCV2-neutralizing antibody titres followed the evolution of the total anti-PCV2 antibody titre. In these pigs, the PCV2-neutralizing antibody titres increased in a time dependent manner until the end of the study. However, one gnotobiotic pig (the pig with high PCV2-replication) and four SPF pigs (pigs that developed PMWS) showed a PCV2-neutralizing antibody profile that significantly differed from their total anti-PCV2 antibody pattern and from the neutralizing antibody pattern observed in the other pigs in the experiment. In the gnotobiotic pig with the high PCV2-replication, no significant PCV2-neutralizing antibodies could be detected using the sensitive SN at any time during the study. The

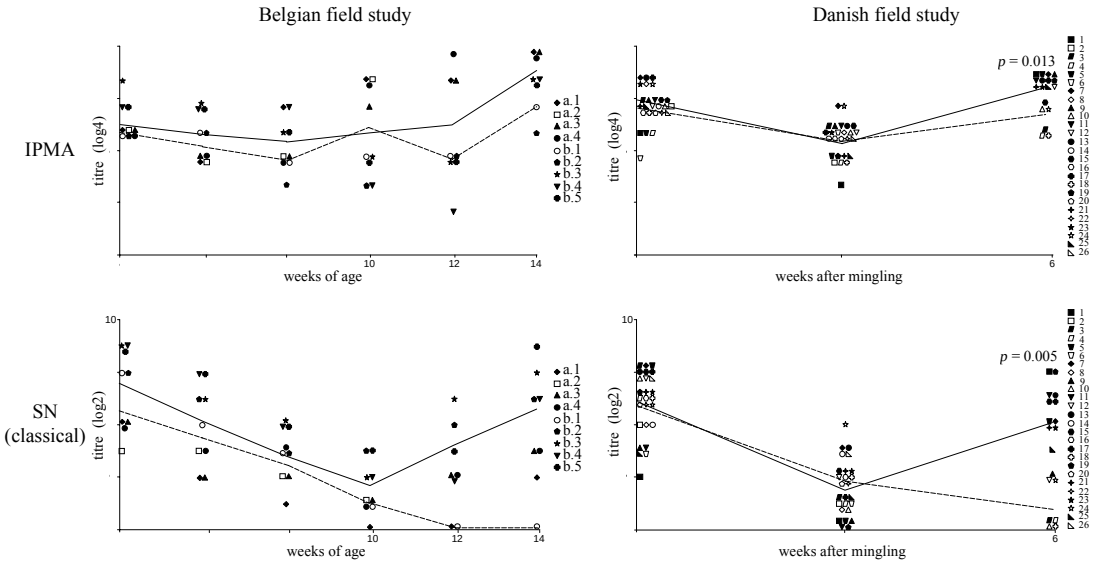
four PMWS-affected SPF pigs showed a peak in PCV2-neutralization at 10 dpi but afterwards the neutralization titres declined until the moment the pigs died (two pigs at 14 dpi) or until neutralization could no longer be detected (2 pigs at 21 dpi). SN-titres in PMWS-affected SPF pigs were significantly lower compared to SN titres in healthy pigs at 14 ($p = 0.011$) and 21 dpi ($p = 0.046$).

Total PCV2-specific and PCV2-neutralizing antibody titres in naturally PCV2-infected pigs

Total PCV2-specific antibody titres (IPMA) and PCV2-neutralizing titres (classical SN) detected in naturally PCV2-infected pigs are presented in Figure 2. All piglets enclosed in both field studies showed maternally derived antibody (MDA) titres between 3.7 and 6.7 log4. A gradual decline of total anti-PCV2 antibodies was detected in all pigs except one (pig 6) in the Danish study. Seroconversion was observed in the Danish pigs between 3 and 6 weeks after mingling and in the Belgian pigs between 6 and 12 weeks of age. In the Danish study, IPMA antibody titres at 6 weeks post mingling were generally lower in PMWS-affected pigs, compared to healthy pigs ($p = 0.013$). However, this difference could not be demonstrated in the Belgian field study.

At the onset of both field studies, SN titres were found above the detection limit with the classical SN in all sera due to the presence of MDA. SN titres at the start of the studies ranged between 4 and 9. At the start of the study, no significant differences in SN Ab titres could be observed between subclinically infected pigs and pigs that developed PMWS. SN titres decreased in a time-dependent manner during the first weeks of the study. In all subclinically infected pigs, except pig 3 in the Danish study, a rise in SN titres could be detected before the end of the study. This seroconversion was detected starting from 10 weeks of age in the Belgian study and starting from 3 weeks after mingling in the Danish study. The majority of the PMWS affected pigs died before they reached the age at which a rise in SN titres could be detected (1 pig in the Belgian and 8 pigs in the Danish study). However 1 PMWS-affected pig in the Belgian study and 5 in the Danish study, survived until the end of the study. In none of these pigs, a rise in SN titres was detected. This resulted in a significant lower SN-titre in the PMWS affected pigs in the Danish study ($p = 0.005$).

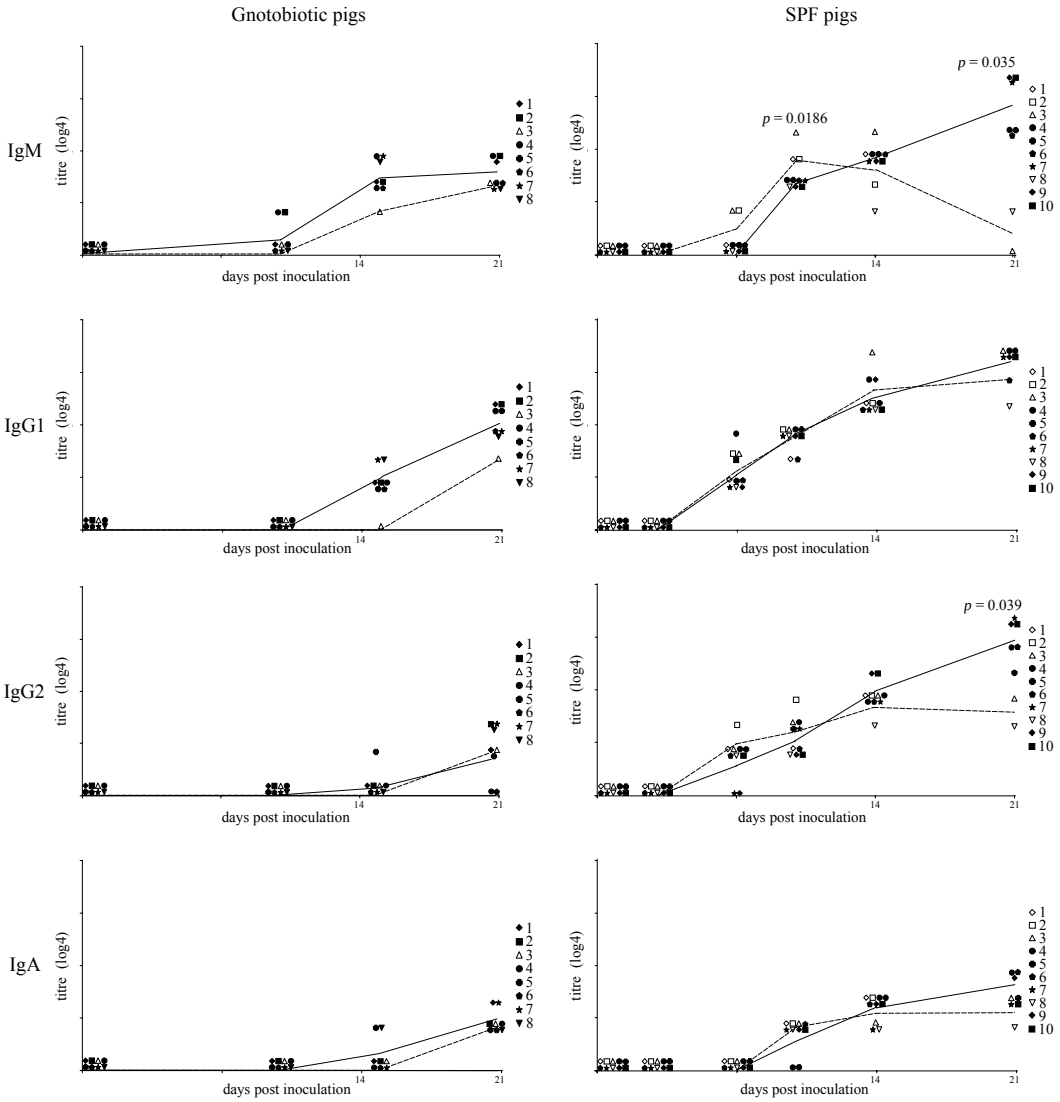
Figure 2. Course of IPMA and SN (Classical) antibody titres in naturally PCV2-infected pigs in Belgian and Danish field conditions. Full symbols: subclinically infected pigs; empty symbols: PMWS-affected pigs. Full line: mean antibody titre of subclinically infected pigs; dashed line: mean antibody titre of PMWS-affected pigs. When difference between mean titres was significant, the *p*-value was shown.



Course of PCV2-specific antibody isotype titres in experimentally inoculated gnotobiotic and SPF pigs

The titres of specific anti-PCV2 antibody isotypes in gnotobiotic and SPF pigs are shown in Figure 3. In gnotobiotic pigs, the different antibody isotypes generally followed the course of the IPMA antibody titres. The IgM, IgG1, IgG2 and IgA titres found in the pig that experienced a high level of PCV2-replication (pig 3) did not differ from the titres of the other pigs. In the pig with high PCV2-replication IgA-type antibodies were detected starting from 15 dpi where as in the other pigs IgA-type antibodies were detected starting from 10 dpi.

Figure 3. Course of isotype-specific antibody titres in experimentally PCV2-inoculated gnotobiotic and SPF pigs. Full symbols: pigs with low PCV2 replication (gnotobiotic) or subclinically infected pigs (SPF); empty symbol: pig with high PCV2-replication (gnotobiotic) or PMWS-affected pigs (SPF). Full line: mean antibody titre of the pigs with low PCV2 replication (gnotobiotic) or healthy pigs (SPF). Dashed line: antibody titre of pig with high PCV2-replication (gnotobiotic) or mean titre of PMWS-affected pigs (SPF). When the difference between the mean titres was significant, the *p*-value was shown.



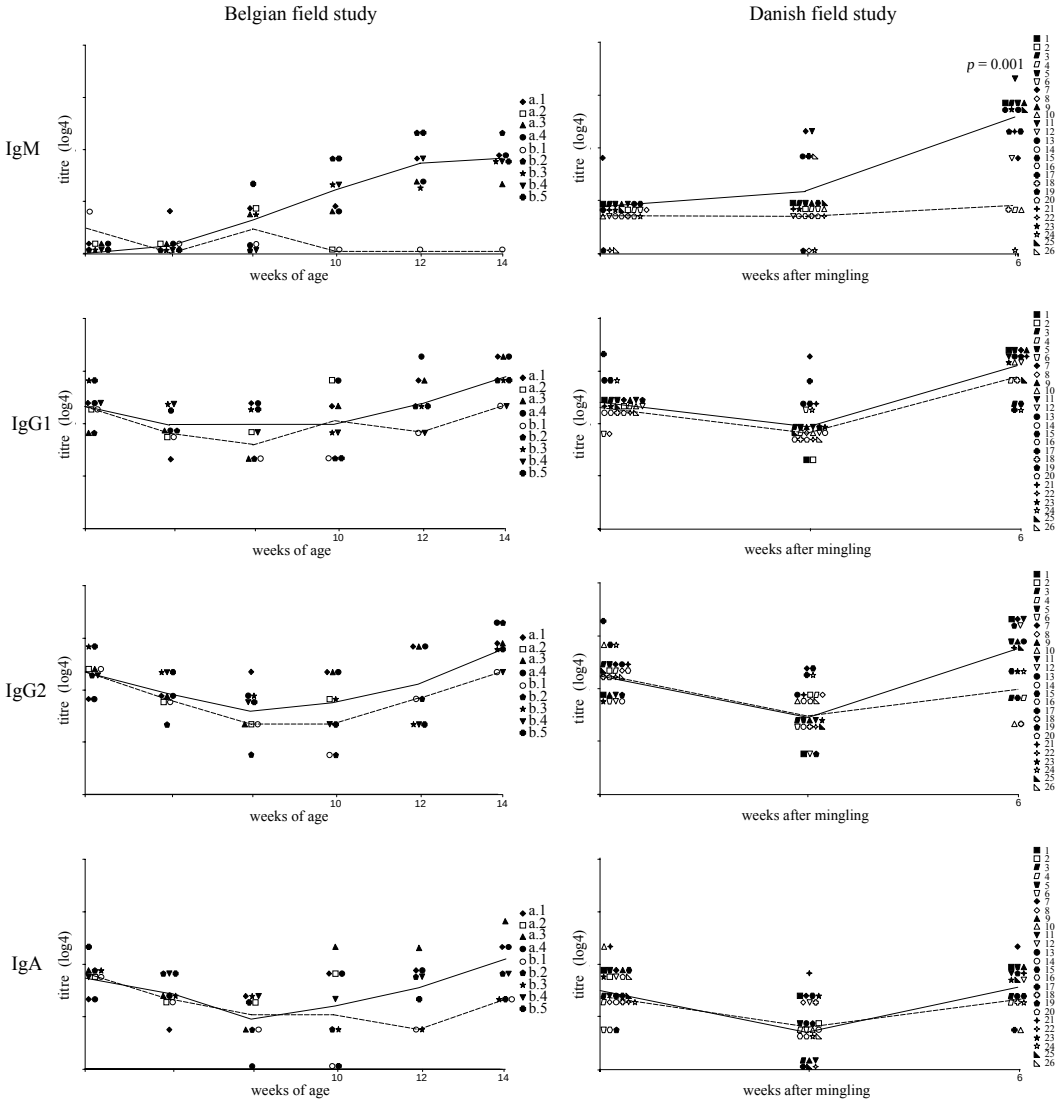
In SPF pigs, a clear difference between PMWS-affected and healthy pigs was observed in the evolution of the IgM-type antibodies. Between healthy pigs, very little variation was detected in IgM titres. Starting from 10 dpi, anti-PCV2 IgM-antibodies were detected and they steadily increased until the end of the study. In 2 out of 4 PMWS-affected pigs, IgM antibodies were detected starting from 7 dpi. At 10 dpi, IgM titres in PMWS-affected pigs were significantly higher than the IgM titres in healthy pigs ($p = 0.018$). After 10 dpi, IgM titres in PMWS-affected pigs deceased towards the end of the study. At 21 dpi, IgM titres in PMWS-affected pigs were significantly lower compared to IgM titres in healthy pigs ($p = 0.035$).

IgG1, IgG2 and IgA titres in SPF pigs generally followed the profiles of the IPMA antibody titres. Titres increased until the end of the study. At 21 dpi, the mean titres of these antibody isotypes in PWMS-affected pigs were lower compared to the titres of the healthy pigs. However, only for the IgG2 titres, these differences were found to be significant ($p = 0.039$).

Course of PCV2-specific antibody isotype titres in naturally PCV2-infected pigs

In naturally PCV2-infected pigs, similar observations were made as in the experimentally inoculated animals. In both field studies, the course of the IgM-type antibodies against PCV2 differed between healthy and PMWS-affected pigs. In healthy pigs, IgM titres were found to increase steadily towards the end of the study, resulting in high IgM titres even at 14 weeks of age, 6 weeks after the first IgM antibodies were detected in some pigs. PMWS-affected pigs had low titres of IgM antibodies. The mean IgM titres in PMWS-affected pigs in the Danish study were significantly lower compared to the titres in healthy pigs ($p = 0.001$). IgG1, IgG2 and IgA titres followed the course of the IPMA antibody titres. It was observed that the mean of these titres in PMWS-affected pigs was always lower compared to the mean titre in healthy pigs. However, these differences were not significant. Due to the limited number of PMWS-affected pigs in the Belgian field study, it was not possible to detect significant differences but it was clear that also in this study the IgM titre in the affected pigs was much lower compared to the titre in their healthy littermates.

Figure 4. Course of isotype-specific antibody titres in naturally PCV2-infected pigs. Full symbols: subclinically infected pigs, empty symbols: PMWS-affected pigs. Full line: mean antibody titre of healthy pigs, dashed line: mean antibody titre of PMWS-affected pigs. When the difference between the mean values was significant, the *p*-value was shown.



Discussion

In the present study, it was shown that the absence of PCV2-neutralizing antibodies was correlated with high PCV2-replication and with PCV2-related disease (PMWS) if present.

Neutralizing antibodies have important functions in the defence of animals and humans against viral infections. Starting from the moment they appear, neutralizing antibodies bind and neutralize the virus and prevent further spread in the body by inhibiting attachment and infection of new cells. It has been shown that neutralizing antibodies against many viruses are more rapidly induced during the immune response and reach higher titres compared to antibodies against other viral epitopes (Bachmann and Zinkernagel, 1997). In contrast, neutralizing antibodies against PCV2 could only be detected starting from 10 dpi by using a sensitive SN assay (Meerts *et al*., 2005). These titres rose slowly until they could be detected with the classical SN assay starting from 4 weeks after inoculation (Pogranichnyy *et al*., 2000). The slow induction of neutralizing antibodies is a very effective mechanism for the virus to ensure a long period of replication in its host. In previous work, the observation was made that an increased replication of the virus in one pig, was correlated with a lack of neutralizing antibody production (Meerts *et al*., 2005). In the present study, it was confirmed that the absence of PCV2-neutralizing antibodies is indeed a reoccurring phenomenon in pigs with high PCV2-replication. In experimentally PCV2-inoculated SPF pigs and in two field studies, a good correlation was observed between the absence or low titres of neutralizing antibodies against PCV2 and the occurrence of a high virus replication and the development of PMWS. This observation reveals a possible mechanism that allows the virus to replicate to a higher extent in a limited number of pigs. These pigs seem to be unable to recognize or produce antibodies against the epitope that is involved in the neutralization of the virus. With relation to other viral infections such as pseudorabies virus (Jacobs and Kimman, 1994), influenza virus (Lambkin and Dimmock, 1995) or human rotavirus (Green *et al*., 1990), it has been shown that different animals do not all recognize the same epitopes. Various syndromes have already been ascribed to the inability of certain individuals to recognize certain epitopes (susceptibility to mastitis (Lunden *et al*., 1990), clinical outcome of Marek's disease virus (Wakenell *et al*., 1996)). The susceptibility to these syndromes is highly genetically determined. The difference in prevalence of PMWS

between regions also leads to the hypothesis that PMWS would be partly genetically determined. Therefore, it would be worth wile to check for a similar mechanism in the recognition of PCV2-neutralizing epitope and the subsequent influence on the susceptibility of the host to PMWS.

In the present study, it was shown that maternally derived antibodies in the serum were able to neutralize PCV2-infection in the SN assay but could not avoid PCV2-infection of the pig since seroconversion was observed in pigs with maternal SN titres as high as 6 log2. When looking at the total antibody titres (IPMA) in the field studies, it was observed that seroconversion against PCV2 never occurred in pigs before weaning at the age of 4 weeks (Belgian field study) or before the pigs were mingled (Danish study). This suggests that none of the pigs got infected with PCV2 as long as they suckled. There are two possible explanations for this. Or the piglets did not get in contact with the virus in the farrowing unit, which is hard to believe since PCV2 is known as a very resistant virus, or piglets were protected from infection as long as they suckled and received lactogenic immunity. This finding may suggest that the primary site of PCV2-replication in the pig is located in the mouth cavity or along the digestive system.

In experimentally inoculated SPF pigs that developed PMWS, a small and transient peak in PCV2-neutralization was observed at 10 dpi. It was observed that these pigs also showed a peak of IgM titres at that same time point. The IgM antibodies in these pigs did not lead to the production of neutralizing IgG or IgA antibodies, which indicates that the neutralizing epitope was not targeted by the humoral immune response. The parallel evolution of the SN and IgM titres in these pigs suggests that the IgM antibodies might be responsible for the low level of PCV2-neutralization that was observed at 10 dpi. Possibly IgM antibodies which are directed against non-neutralizing epitopes, are able to neutralize PCV2 to some extent by sterical hindrance. Indeed, IgM antibodies are secreted in a pentamere formation that is much larger compared to any other antibody isotype. This pentamere form contains 10 Fab fragments which would enable it to bind PCV2 at different sites. In PMWS-affected pigs in the Danish field study, a peak in IgM antibodies was not detected probably because the time intervals between the different blood collections were too long (3 weeks). When comparing IgM profiles in both experimental studies, it is observed that in the gnotobiotic pig with high PCV2-replication, low IgM titres were detected until the end of the study. In general the humoral immune response in

gnotobiotic pigs was delayed compared to the SPF pigs, which might be the reason that no decrease in IgM titres was observed in this pig. The low IgM titres in this gnotobiotic pig might correspond to the slight increase in PCV2-neutralization (up to 16% whereas in other pigs they reached 97%) that was observed in this pig. This slight neutralization however, was not significantly different compared to the result obtained from serum of the same pig before inoculation.

IgM titres in subclinically infected animals, in contrast with titres in PMWS-affected pigs, persisted and even increased until the end of the study. After a viral infection, IgM-type antibodies are the first antibodies that are being produced. Generally, the plasma cells that produce these antibodies go through an isotype switch and start producing other isotypes of antibodies resulting in a down-regulation of IgM production (Esser and Radbruch, 1997). A persistence of IgM type antibodies has already been described during the immune response against other viruses such as the coronavirus responsible for the severe acute respiratory syndrome in humans (Woo *et al*., 2004) or the human parvovirus B19 (Yeagashi *et al*., 1989). However, the mechanism of this prolonged presence of IgM antibodies has not been defined yet. It is known that in older animals such as breeding sows, very high IPMA antibody titres against PCV2 can be found. It may be hypothesized that the persistent presence of IgM lies at the base of this observation.

Although a clear difference in the course of the IgM antibodies was observed between healthy and PMWS-affected pigs, the isotype switch occurred in both kinds of pigs at the same time point since other antibody isotypes were detected starting from the same time point. In PMWS-affected pigs, titres of other antibody isotypes against PCV2 were somewhat lower compared to titres in healthy pigs at the end of the studies. These differences, although hard to explain, indicate that some important changes in the humoral immune response occur. Possibly they are related to the fact that PCV2 is able to infect B-cells and causes a severe depletion of especially the B-cells (Sanchez *et al*., 2004; Nielsen *et al*., 2003). Whether these changes are important in the induction of PMWS or are merely a consequence of it, remains unclear.

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6.

General discussion

With regard to their structure, circoviruses are without doubt the most uncomplicated viruses that are known today. Their exceptionally small genome codes for only one structural protein (Nawagitgul *et al*., 1998) and two replication-associated non-structural proteins. Both non-structural proteins are actually being transcribed from one open reading frame (Mankertz and Hillebrand, 2001), again compressing the amount of DNA needed to enclose all the genetic information that is required for the replication of the virus. The fact that such rudimentary viruses are able to infect eukaryotic cells and complete their own replication, is an amazing achievement. Except for prions (Stahl and Prusiner, 1991), circoviruses are probably the smallest entities that are able to reproduce themselves or better, that are able to get themselves reproduced. Circoviruses are not only able to successfully multiply in an individual host, they are also very successful in maintaining themselves in the population of their respective hosts. This can be deduced from the high prevalence of the porcine circoviruses (Labarque *et al*., 2000), TT-virus (Ali *et al*., 2002) and psittacine beak and feather disease virus (Rahaus and Wolff, 2003).

Coexistence of viruses and their hosts inflicts an evolutionary pressure on both host and virus (Vossen *et al*., 2002). As such, it can be hypothesized that the establishment and optimization of the immune system of higher organisms, is an ever-improving answer to external threats posed by infectious agents (viruses, bacteria and parasites). The immune system is especially designed to decrease the chance of survival of these infectious agents in the population of their host. When the structure of contemporary viruses is overviewed, it becomes clear that viruses have evolved in different directions to cope with this immune system. Viruses of different orders can follow quite opposed strategies to fight against the onset and their elimination by their host's immune response.

One strategy is used by structurally more complex viruses. Through evolution, such viruses have developed mechanisms that enable them to actively avoid recognition or disturb their host's immune response. These different viral immune evasion strategies have been exhaustively reviewed in literature (Tortorella *et al*., 2000; Vossen *et al*., 2002). The implementation of such mechanisms requires the presence of a complex machinery of viral proteins that has to be carried in the genome of the virus or in some cases in the virion itself. As a consequence, the virus will become more complex as it adapts to various host mechanisms.

Structurally more simple viruses such as the circoviruses can follow an opposite strategy. By expressing so few viral proteins, circoviruses supply their host with a very limited number of epitopes to initiate its immune response against. As a consequence, the way for circoviruses to circumvent the onset of an efficient immune response is to avoid recognition. As far as it is known today, PCV2 only expresses three proteins and in this thesis as well as in a previous publication, it was shown that no antibodies against two of these three proteins, the Rep proteins, could be demonstrated (Cheung and Bolin, 2002; Meerts *et al*., 2005a). This leaves only the capsid protein as a possible target for the humoral immune response. Unfortunately, also the recognition of the capsid protein is not optimal in all pigs.

In this thesis, the influence of the specific immune response on the development of a PCV2-infection in a pig was clarified. It has been shown as well in this thesis as by other researchers (Ladekjær-Mikkelsen *et al*., 2002; Sanchez *et al*., 2003) that, upon experimental inoculation, piglets with identical backgrounds experience different evolutions in the replication of PCV2. In the majority of the pigs, a peak in PCV2-replication was observed between 10 and 15 days post inoculation, followed by a gradual decrease of the virus titre. This decrease coincided with the onset of an efficient specific immune response that consisted of the production of PCV2-neutralizing antibodies and in some cases also of a clearly detectable cellular immune response. Similar replication kinetics have already been observed after experimental inoculation with many other viruses (Glass *et al*., 2004; Labarque *et al*., 2000b; Lichtenstein *et al*., 1995). However, in a number of PCV2-inoculated pigs, the replication of the virus continued to increase after the time point at which the other pigs reached their peak in PCV2-replication, resulting in considerably higher titres in these pigs at the end of the study. It could be demonstrated that in gnotobiotic pigs experiencing this pattern of PCV2-replication, the cellular immune response was too low to be detected and more important, that no PCV2-neutralizing antibodies were detected (Meerts *et al*., 2005b). This inefficient immune response clearly rendered the pigs more susceptible to an increased level of PCV2-replication since the virus was able to replicate in these pigs as good as it did in pigs that were artificially immunosuppressed by a treatment with Cyclosporin A. These results indicate that the specific immune response in general, and more specific the absence of PCV2-neutralizing antibodies, is the determining factor for the virus to go into the direction of a prolonged replication.

The economic relevance of PCV2 lies in its association with the postweaning multisystemic wasting syndrome (PMWS), a disease which is generally observed in pigs a few weeks after weaning. The morbidity in field situations varies between 0.01% (Meerts *et al.*, 2004) and 30% (Allan and Ellis, 2000). In most cases, the disease affected a small fraction of the pigs. The association between PCV2 and this disease was made after the observation that PMWS-affected pigs all contained high titres of PCV2 in various organs. Only low titres of the virus or no virus could be isolated from healthy pigs that were sacrificed at the time at which litter mates died of PMWS (Meerts *et al.*, 2004). During several experimental inoculations in pigs in which PMWS was reproduced, slight differences in seroconversion against PCV2 were observed in-between pigs and correlated with the appearance of PMWS: PMWS-affected pigs tended to have lower total antibody titres against PCV2 (Ladekjær-Mikkelsen *et al.*, 2002). Furthermore, it has been noted in several studies that pigs, when developing PMWS, seroconvert at later time points compared to subclinically infected pigs (Bolin *et al.*, 2001; Rovira *et al.*, 2002; Okuda *et al.*, 2003). The mechanisms that form the base of these differences were not fully understood. However, they are indicative for some modulations of the humoral immune response in pigs that develop PMWS. In the work presented in this thesis, it was shown that the absence of PCV2-neutralizing antibodies was a recurring phenomenon in pigs with high PCV2-replication (Meerts *et al.*, 2005b; Meerts *et al.*, 2005d). Thereby, it was demonstrated that the absence of PCV2-neutralizing antibodies coincided not only with a prolonged replication of the virus, but also with the development of PCV2-related disease (Meerts *et al.*, 2005d).

Although pigs with a high level of PCV2-replication did not produce PCV2-neutralizing antibodies, they did show a normal seroconversion when looking at their total anti-PCV2 antibodies. This indicates that these pigs produced antibodies against PCV2 but not against a specific epitope that is involved in the antibody-mediated neutralization of the virus. With regard to other viruses (pseudorabies virus, type A influenza virus, rotavirus), it has already been shown that not all hosts develop antibodies to the same extent to all different viral epitopes (Jacobs and Kimman, 1994; Lambkin and Dimmock, 1995; Green *et al.*, 1990). Most viruses contain a high number of epitopes and chances are relatively low that the inability of an animal to recognize one of these epitopes may have severe consequences. When a similar event occurs with a very small virus such as a circovirus, chances are much higher that not

recognizing one of the few available epitopes may have severe consequences on the efficiency of the immune system to eliminate the virus.

Several syndromes in various animal species have already been associated with the inability of certain animals to recognize a specific epitope of an infectious agent (Lunden *et al*., 1990; Wakenell *et al*., 1996). The mechanism that is responsible for this event in these syndromes has been identified. It has been shown that the major histocompatibility complex class II (MHC-II) is highly polygenic and polymorphic. This high diversity allows antigen presenting cells to present as many "non-self" epitopes as possible through their MHC-II molecules, in order to initiate an immune response against them (Kumanovics *et al*., 2003). However, when an individual lacks the MHC-II haplotype that is responsible for the presentation of a certain epitope, it will not react against that epitope. As such it has been described that in chickens, the B19 haplotype is associated with susceptibility to Marek's disease whereas the B21 haplotype is associated with resistance against the disease when they are infected with the virus (Wakenell *et al*., 1996). In bovines, the MHC II haplotype DQ1A was associated with an increased susceptibility to clinical mastitis (Lunden *et al*., 1990). It would therefore be very interesting to identify the epitope on the PCV2 capsid protein that is involved in the neutralization of the virus and subsequently to identify the MHC-II haplotype that is responsible for presenting this epitope. The MHC-II genes are inherited from the parents. Diseases that are linked with specific genotypes are highly genetically determined. Related to the field, this results in the existence of susceptible and resistant breeds or lines. If a similar mechanism would be at the base of the susceptibility of certain pigs to an increased PCV2-replication and subsequently to PMWS, this would immediately confirm and explain the presence of a genetical influence on the prevalence of PMWS. In the past, it has already been shown that the genetic background of pigs has an influence on their susceptibility to PMWS (personal observations; Lopez-Soria *et al*., 2004). However, the search for the underlying mechanism has only recently been initiated. The results obtained in this thesis might lead the way to a successful identification of this mechanism.

Our results indicate that the humoral immune response is of major importance in the pathogenesis of the PCV2-infection. When this humoral immune response was studied in more detail, some more interesting differences between subclinically infected and PMWS-affected pigs were found. A general trend was that PMWS-affected animals showed lower total antibody titres upon infection. The differences

between the mean titres of PMWS-affected and subclinically infected animals however, were not always significant. It indicates that the total antibody titres were only slightly decreased in animals affected with PMWS. This observation had been made previously in other studies (Ladekjær-Mikkelsen *et al*., 2002; Sanchez *et al*., 2003). A more obvious difference was observed in the evolution of the IgM-titres between the subclinically infected pigs and pigs that developed PMWS. During a "normal" humoral immune response against a viral infection, IgM antibodies are the first antibodies that can be detected. After a short period, the plasma cells that produce these antibodies experience an isotype switch that results in the production of other isotypes of antibodies and in the decrease of IgM production (Esser and Radbruch, 1990). This property of the IgM antibodies has therefore been used in assays to diagnose a recent infection of a virus in a single serum sample (Vasquez *et al*., 2005; Sagnelli *et al*., 2005). This profile of transient IgM-production was observed in pigs that developed PMWS. In the healthy pigs, IgM antibodies remained detectable and even increased until the end of the studies at 21 days after PCV2 inoculation. From these results, it looks like PMWS-affected pigs show the "normal" IgM profile, whereas healthy pigs show an "abnormal" profile. Some other viruses are also known to cause a persisting IgM production in their host. The coronavirus causing the severe acute respiratory syndrome in humans (Woo *et al*., 2004) as well as the human parvovirus B19 (Yaegashi *et al*., 1989) induce IgM titres in serum that can be detected during several months. The mechanism that leads to these persistent IgM antibodies is unknown. From the results in this thesis, it could not be concluded whether this obvious difference in IgM production profiles between subclinically infected and PMWS-affected pigs was the cause of the development of disease or merely a consequence of it. The isotype switch of antibodies is highly controlled by different cytokines (Kawanishi *et al*., 1991; Fontana et al., 1992; Gao *et al*., 2001). Recently, *in vivo* observations made by other research groups have suggested an influence of certain cytokines on the replication of the virus and the susceptibility to the disease (Hasslung *et al*., 2005). In this thesis, some indications were found that this might indeed be the case.

One event that will undoubtedly have an impact on the evolution of the humoral immune response in pigs with an increased PCV2-replication is the fact that in such pigs, B-cells have been found to be major target cells of the virus. Furthermore, high PCV2-titres have been correlated in many studies with depletion of lymphocytes and

especially with B-cells. This was observed in lymphoid tissues (Sanchez *et al*., 2004) as well as in the blood (Nielsen *et al*., 2004). This event might be responsible for the slightly lower total anti-PCV2 antibody titres that have been found in PMWS-affected pigs.

So far, it has been shown in this thesis that the replication of PCV2 is highly influenced by the host's immune response against PCV2. The full identification of the mechanism behind the observed differences is important in understanding the inherent susceptibility of an individual pig or a certain breed of pigs to sustain a high level of PCV2-replication and subsequently develop PCV2-associated diseases. If these mechanisms would be genetically determined, the pig industry would for instance be able to select pig breeds that are resistant to high PCV2-replication and associated diseases.

Besides being influenced by the host itself, the course of a PCV2-infection in pigs can be influenced by many external factors. One of the earliest observations made during the investigation on the pathogenesis of a PCV2-infection in pigs, was that a co-infection of pigs with porcine parvovirus (PPV) (Allan *et al*., 2000a) enhanced the replication of PCV2. Thus, PPV created an environment in the host that was favourable for PCV2-replication. This was not a unique property for PPV alone. Similar experiments with the porcine reproductive and respiratory syndrome virus (PRRSV) resulted in a comparable effect on the replication of PCV2 in the pig (Allan *et al*., 2000b). In an attempt to find the mechanism behind the enhancement of PCV2-replication by these co-infections, gnotobiotic pigs were inoculated with keyhole limpet hemocyanin in Freund's incomplete adjuvant. This treatment also increased the susceptibility of pigs to an increased PCV2-replication (Krakowka *et al*., 2001). A common event in all these co-infections or treatments is the induction of an immune response. Unfortunately, research focussed mainly on the identification of other potential cofactors that were able to enhance PCV2-replication (bacterins, vaccines, *Mycoplasma Hyopneumoniae*) (Opriessnig *et al*., 2003; Opriessnig *et al*., 2004a; Opriessnig *et al*., 2004b). The mechanism that was responsible for this increased PCV2-replication remained to be identified.

The initiation of a specific immune response by an infectious agent implies the production of a wide array of different cytokines. Many of these cytokines can be detected in the blood, indicating that they are distributed throughout the body where they induce systemic effects. A number of the cytokines that are produced during an

immune response have been found to influence the course of viral infections in general in the host. Tumour necrosis factor alpha (TNF-α) (Calabrese *et al*., 2004) and certainly the different types of interferons (Katze *et al*., 2002), inhibit the replication of many viruses both *in vitro* and *in vivo*. The antiviral effect of interferon-alpha (IFN-α) has been found to be so consistent that recombinant protein is used to treat various viral infections in humans and animals (Maggi *et al*., 2001; Martin *et al*., 2002). The question rose how it is possible that PCV2 is able to replicate better in pigs coinfected with PPV or PRRSV, knowing that such infections induce these cytokines with antiviral properties. Therefore the effect of a range of porcine cytokines on the replication kinetics of PCV2 in continuous cell lines was investigated in this thesis.

First, the replication kinetics of PCV2 was established in non-treated PK-15 cells (Meerts *et al*., 2005a; Misinzo *et al*., 2005). In this study, it was observed that the completion of the full replication cycle of PCV2 took up to 24 hours, which is very long compared to that of other viruses. During the first stages of the infection, the structural capsid protein was produced and remained present in the cytoplasm of the cell. Subsequently, the replication-associated Rep protein was produced and both capsid and Rep protein were relocated to the nucleus to form the infectious virions. These observations agreed with the sequence of events that have been described during the replication cycle of viruses that follow the rolling cycle replication (Koths and Dressler, 1980). A striking result was obtained when these cells were treated with IFN-α or IFN-γ. It was demonstrated that IFN-γ and, in more specified conditions, also IFN-α were able to enhance the replication of the virus. This was the first description of an enhancement of any viral infection by IFN-α and IFN-γ. The enhancing effect was mainly due to an increased internalization of the virus into the cells to which it was bound. This finding reflects a possible mechanism of major importance to understand the interaction between PCV2 and the cell it infects but also between the virus and the immune system of its host (Meerts *et al*., 2005c).

Although this finding could not be reproduced *in vivo* by injecting pigs with recombinant IFN-γ (rIFN-γ), an enhancement of the replication of PCV2 in pigs could be induced with concanavalin A (ConA) (Meerts *et al*., 2005e). This led to two different hypotheses, (1) the effect of the ConA-treatment on the replication of PCV2 was at least partially IFN-γ dependent but not reproduced with rIFN-γ alone because

the obtained concentrations of IFN-γ in the blood after injecting the pigs with rIFN-γ were too low or (2) the effect of ConA was IFN-γ independent. Nevertheless, this experiment showed that stimulation of T-cells, which is a normal event during an immune response, enhanced the replication of PCV2 in the pig. Thus, this mechanism might be the cause of the increased PCV2-replication in immunostimulated pigs.

Both stimulation of T-cells by ConA-treatment and inhibition of T-cell proliferation by CyclosporinA (CysA) induced a similar effect. The difference between both treatments was that ConA did not only induce higher PCV2 titres compared to the control pigs, it also sped up the replication since PCV2 could be detected in the lymph nodes of the pigs starting from an earlier time point. Thus, ConA seemed to increase the replication of the virus also in the early stage of the infection whereas CysA increased the replication merely by inhibiting the specific immune response during the late stage of the infection. Nevertheless, these data reflect the unique interaction between PCV2 and the immune system of its host.

This thesis aimed to identify factors that are able to influence the replication of PCV2 in its host. It was shown that the efficiency of the cellular and more importantly of the humoral immune response had an influence on the development of the PCV2-infection and subsequently on the pig's susceptibility to PCV2-related disease. Additionally, a mechanism was proposed by which PCV2 could be able to use the immune response against another infectious agent to improve its own replication. Thus, the evolution of the replication of PCV2 in a pig and its clinical outcome is the result of various host specific and external factors of which a few have been identified. Further studies on the pathogenesis of a PCV2 infection will therefore always have to consider the influence of these factors. Any disease that is associated with PCV2 is thus, by definition, a multifactorial disease and neither prevention nor treatment of such a disease should focus on PCV2 alone.

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7.

Summary/Samenvatting

Summary

Porcine circovirus type 2 (PCV2) is a virus that has been associated with a condition in pigs known as the postweaning multisystemic wasting syndrome (PMWS). Upon experimental inoculation or natural infection, the replication of the virus shows an unusual high variation between individual pigs. This variation indicates that the replication of the virus in the pig is influenced by unknown factors. In literature, indications can be found that some of these factors are related with the immune system of the animal. The present thesis aimed to identify host specific and external factors that are able to influence the replication of the virus in the pig.

In the introduction in chapter 1, an overview was given on the classification and the structure of the circoviruses. The current knowledge of the pathogenesis of a PCV2-infection in a pig and its association with various disease syndromes in pigs was reviewed.

Chapter 2 gives the aims of the thesis. These aims were first to establish the replication kinetics of PCV2 in continuous cell lines and in its most important target cells. Afterwards it was investigated if a range of different cytokines were able to influence the replication of PCV2 *in vitro* and *in vivo*. Another aim was to study in detail the immune response upon a PCV2-infection and to correlate the onset of this immune response with the further development of the infection and with the occurrence of PCV2-related disease.

Chapter 3 presents a study that was initiated to compare the replication kinetics of different strains of PCV2 in the continuous porcine cell line PK-15 and two primary porcine cells that were found to be major target cells *in vivo*: porcine alveolar macrophages (PAM) and fetal cardiomyocytes (FCM). The aim of this study was to compare the replication kinetics of PCV2 in the different cell types and to investigate whether biological differences could be found between PCV2 strains that were isolated from pigs with different PCV2-related syndromes. In a first study, PK-15, PAM and FCM cultures were inoculated with either the postweaning multisystemic wasting syndrome (PMWS)-associated strain Stoon-1010 or the abortion-associated strain 1121. Viral proteins were visualized by immunofluorescence stainings and

virus production was determined by titrating the culture supernatant on porcine circovirus-free PK-15 cells. In PK-15 cells, the capsid protein was expressed between 6 and 12 hours post inoculation (hpi), it relocated to the nucleus between 12 and 24 hpi. Starting from 24 hpi, the replication-associated Rep protein was also detected in the nucleus. During the early stages of the infection, Rep protein could not be detected in the cytoplasm of the infected cell. Only in PK-15 cells that were dying (condensed and fragmented nucleus) some Rep protein could be found in the cytoplasm, most probably due to leakage from the disorganized nucleus. A similar sequence of events also occurred in the primary cell lines FCM and PAM, but nuclear localized antigens appeared at a later time point (48 hpi) and in a lower percentage of cells. In PAM, clear differences in susceptibility were seen between pigs. In PAM from two out of five tested pigs, no nuclear localized antigens could be detected, whereas in PAM from three other pigs they were seen in up to 20% of the antigen-positive cells. Virus production was observed in PK-15 but not in PAM or FCM cultures. In a second study, the replication kinetics of seven different PCV2 strains were compared in PK-15 cells. It was shown that 2 abortion-associated strains had different replication kinetics in comparison with PMWS or porcine dermatitis and nephropathy syndrome associated strains. With the abortion-associated strains, a higher number of infected cells was observed after 24 hours, the fraction of these cells with nuclear localized antigens was lower compared to the other strains. From this study, it could be concluded that the replication kinetics of PCV2 in the primary cells PAM and FCM was similar but slower compared to the replication kinetics in PK-15 cells. Furthermore, it was observed that not all batches of PAM were equally susceptible to PCV2. This observation possibly reflects that the state of the macrophages in the pig influences the susceptibility of the pig to PCV2-infection. The differences in the replication kinetics between the different PCV2 strains indicates that some biological differences may occur even though the strains are genetically very similar.

Stimulation of the porcine immune system results in an increased replication of porcine circovirus 2 (PCV2) *in vivo*. Since it is known that a stimulation of the immune system coincides with the production of various cytokines, a study was performed to investigate the influence of certain cytokines on the infection and replication of PCV2. Chapter 4 illustrates particularly the effect of type I and type II interferons on the replication of PCV2 *in vitro* and *in vivo*. In chapter 4.1 it was

investigated if a range of cytokines (interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor-alpha (TNF-α), interferon-alpha (IFN-α) and interferon-gamma (IFN-γ)) were able to influence the course of a PCV2-infection *in vitro*. No changes were observed in IL-1, IL-6, TNF-alpha or IL-10 treated cells. However, it was demonstrated that IFN-α and IFN-γ influenced PCV2 infection in porcine kidney cells (PK-15) and porcine monocytic cells (3D4/31). IFN-γ added to the culture medium before, during or after inoculation increased the number of PCV2-antigen positive cells with 418, 171 or 691% in PK-15 cells and with 706, 114 or 423% in 3D4/31 cells. IFN-α pre-treatment decreased the number of infected PK-15 cells. However, when it was added after inoculation, it enhanced PCV2 infection with 529% in PK-15 cells and 308% in 3D4/31 cells. The effect of both interferons on PCV2 infection was dose-dependent and could be blocked with their respective neutralizing monoclonal antibody. Leukocyte-derived porcine IFN-γ induced a similar effect on PCV2-infection. Treatment of PK-15 cultures with IFN-γ resulted in a 20 times higher production of progeny virus. Confocal microscopy studies showed that the enhancing effect of IFN-γ on PCV2-infection was achieved by increased internalization of PCV2 virion-like particles (VLPs). Binding of the VLPs to the cell or expression kinetics of PCV2-proteins in infected cells, were not altered by IFN-γ treatment. To our knowledge, this study reported the first enhancement of a viral infection by treatment with type I and type II interferons.

In chapter 4.2 an attempt was made to reproduce the findings made in chapter 4.1 *in vivo*. In this study, gnotobiotic pigs were intramuscularly and subcutaneously injected with Concanavalin A (ConA) or recombinant porcine IFN-γ (rIFN-γ) 12 hours before experimental PCV2-inoculation and every 3 days for 9 days during the infection. ConA is a lectin that activates Th1, this goes along with the production of IFN-γ by these cells. The replication of PCV2 was monitored at 3 different time points in individual pigs by taking lymph node biopsies. The evolution in PCV2-replication was determined by titrating the amount of PCV2 in the lymph node biopsies. In non-treated control pigs and rIFN-γ inoculated animals, PCV2 could be detected starting from 15 days post inoculation (dpi). No significant differences in the level of PCV2-replication between pigs from these different groups were found. In gnotobiotic pigs treated with ConA, PCV2 could already be isolated from inguinal lymph nodes starting from 10 dpi. At 15 dpi, the level of PCV2-replication was

significantly higher in ConA-treated pigs compared to the non-treated group. Although ConA treatment significantly increased PCV2-replication, no signs of disease were observed in any of the inoculated or non-inoculated animals. These experiments indicate that ConA-treatment enhances the replication of PCV2 *in vivo*, however data from the rIFN-γ treated pigs were not conclusive to show that the ConA-mediated enhancement of PCV2-replication was IFN-γ mediated.

In chapter 5, the immune response of pigs against PCV2 was explored. Chapter 5.1 describes the findings that were made in a study in which the cellular and humoral immune response were monitored in individual gnotobiotic pigs and correlated with the evolution of the replication of the virus. Twelve gnotobiotic pigs were inoculated with PCV2. Four of these pigs were treated with Cyclosporin A (CysA) to monitor the effect of the adaptive immunity on the development of the PCV2-infection. Through lymph node biopsies at 10, 15 and 21 days post inoculation (dpi), PCV2-replication in lymphoid tissues was monitored. The production of total PCV2-specific and PCV2-neutralizing antibodies was followed, together with interferon-gamma (IFN-γ) mRNA expression levels in peripheral blood monocytes as a marker for cellular immunity. Generally, the CysA-treated pigs showed the highest PCV2-titres, indicating that the adaptive immunity is necessary to restrain PCV2-replication. Three different PCV2-replication patterns were observed in non-CysA-treated pigs. Pattern 1: in two pigs, PCV2 was not detected. They had the highest neutralizing antibody titres, appearing from 15 dpi. In these pigs a good cellular response was indicated by a peak in IFN-γ mRNA at 15 dpi. Pattern 2: five pigs contained low to moderate PCV2 titres at 15 dpi, remaining constant or decreasing towards 21 dpi. Lower neutralizing antibody titres were observed and no rise in IFN-γ was detected. Pattern 3: in one pig, a low PCV2-titre at 15 dpi dramatically increased towards 21 dpi. Although an antibody-response against PCV2 was mounted, no PCV2-neutralizing antibodies were detected. This pig also showed no rise in IFN-γ mRNA. The study indicates that variation in the onset of the adaptive immunity may account for variation in PCV2-replication between pigs. Absence of PCV2-neutralizing antibodies may be an important factor in the development of an increased virus replication. However, this study was performed in gnotobiotic pigs and it has been shown that the immune response of these pigs may deviate from the response of conventional animals. Therefore, in chapter 5.2 it was

investigated if similar observations could be made in experimentally or naturally PCV2-infected conventional animals. In the study presented in this chapter, it was shown that the correlation between the absence of PCV2-neutralizing antibodies and a high replication of the virus could also be found in SPF pigs in which PMWS was experimentally reproduced and in naturally PMWS-affected pigs. When looking at the total anti-PCV2 antibodies, PMWS-affected and healthy animals seroconverted at the same time but titres were slightly lower in PMWS-affected animals. In healthy animals the evolution of PCV2-neutralizing antibodies followed that of the total anti-PCV2 antibodies whereas in PMWS-affected animals seroconversion in neutralizing antibodies was not observed (field studies) or they were detected only in low concentrations for a period of 1 week after which they disappeared (experimentally inoculated SPF pigs). Differences were found between the evolution of specific antibody isotypes against PCV2 in PMWS-affected and healthy pigs. In healthy pigs, IgM antibodies persisted until the end of the study whereas in PMWS-affected pigs they quickly decreased or remained at low titres. Furthermore, the mean titres of all other antibody isotypes tested (IgG1, IgG2 and IgA), were slightly lower in PMWS-affected pigs compared to their healthy group mates at the end of each study. These differences were not always significant.

A general conclusion of this thesis is that the level of PCV2-replication in a pig can be influenced by many host specific and external factors. It was demonstrated that in several experimental and field studies a small proportion of the pigs were not able to initiate an efficient immune response against the virus since they did not produce PCV2-neutralizing antibodies. This resulted in an increased level of PCV2-replication in these pigs and the development of PMWS in some studies. This inability of a proportion of the pigs to produce PCV2-neutralizing antibodies might be the clue to understand the genetical background of the susceptibility of pigs to PMWS. In this thesis another factor of possible importance was identified. It was demonstrated that IFN-γ and in more specific conditions also IFN-α were able to increase the susceptibility of porcine cell lines to PCV2-infection. A similar mechanism *in vivo* may explain the enhancement of PCV2-replication that has been observed in pigs when an immune response is induced in the pigs. Further work based on this thesis might supply pig producers with strategies to select towards a breed of pigs that is

resistant to high PCV2-replication or to implement sanitary measures to minimize the risk of inducing high PCV2-replication in the present pig population.

Samenvatting

Het porcien circovirus type 2 (PCV2) is een zeer klein en vrij eenvoudig virus met een enkelstrengig, circulair DNA. Het wordt geassocieerd met een syndroom bij biggen dat gekend is als het infectieus groeistopsyndroom. Dit syndroom is echter beter bekend onder zijn Engelstalige naam: postweaning multisystemic wasting syndrome (PMWS). Na experimentele inoculatie van het virus bij biggen of na een natuurlijke infectie, vertoont de vermeerdering van het virus een uitzonderlijk hoge variatie. Deze hoge variatie toont aan dat de vermeerdering van het virus in het big beïnvloed wordt door bepaalde co-factoren die totnogtoe onvoldoende gekend zijn. In de wetenschappelijke literatuur kunnen indicaties gevonden worden dat deze factoren verband houden met het immuunsysteem van de dieren. Er werd namelijk aangetoond dat het induceren van een immuunrespons in dieren die reeds geïnfecteerd zijn met PCV2, aanleiding gaf tot een verhoogde vermeerdering van PCV2. In het verleden werd deze immuunrespons geïnduceerd door de dieren te inoculeren met bijkomende virussen of bacteriën, door ze te vaccineren of door een sterk immunogene stof te injecteren. De doelstellingen van deze thesis waren om zowel gastheerspecifieke als externe factoren te identificeren die in staat zijn om de vermeerdering van PCV2 te verhogen in het big.

In de inleiding in hoofdstuk 1 wordt de classificatie en de organisatie van de circovirussen besproken. Verder wordt een overzicht gegeven van de bestaande kennis over de pathogenese van een PCV2-infectie en van de verschillende syndromen waarmee het virus reeds geassocieerd wordt.

Hoofdstuk 2 geeft de doelstellingen van de thesis weer. De eerste doelstelling was om de vermeerderingskinetiek van PCV2 in continue cellen en in zijn belangrijkste doelwitcellen te onderzoeken. Nadien werd uitgezocht of bepaalde cytokines deze vermeerderingskinetiek konden beïnvloeden. De invloed van deze cytokines werd zowel *in vitro* als *in vivo* onderzocht. Een andere belangrijke doelstelling in deze thesis was om de specifieke immuunrespons tegenover PCV2 in geïnfecteerde biggen te onderzoeken en na te gaan in hoeverre deze immuunrespons verantwoordelijk was voor het verdere verloop van de infectie en eventueel een rol

speelde in de gevoeligheid van de dieren voor ziekten die met PCV2 geassocieerd worden.

Hoofdstuk 3 geeft de resultaten van een studie weer waarin de kinetiek van de vermeerdering van verschillende PCV2 stammen werd vergeleken in de continue varkensniercellijn PK-15 en in twee primaire cellijnen waarvan gekend is dat ze bij het big belangrijke doelwitcellen zijn: porciene alveolaire marcofagen (PAM) en foetale cardiomyocyten (FCM). De doelstelling van deze studie was enerzijds om de vermeerderingskinetiek van twee referentiestammen te vergelijken in de verschillende cellijnen om te onderzoeken of er een verschil in gevoeligheid tussen de cellijnen was, anderzijds om te onderzoeken of er biologische verschillen optraden tussen PCV2 stammen die geïsoleerd werden uit varkens aangetast door verschillende syndromen. In een eerste studie werden PK-15 cellen, PAM of FCM geïnoculeerd met de PMWS-geassocieerde stam Stoon-1010 of met de abortus-geasocieerde stam 1121. Op verschillende tijdstippen na de inoculatie werden de cellen gefixeerd en geanalyseerd. Virale eiwitten werden gevisualiseerd door middel van een immunofluorescentie kleuring en de virusproductie werd bepaald door het virus in het supernatans van de celculturen te titreren in porcien circovirus-negatieve PK-15 cellen. Het kapsied eiwit werd in PK-15 cellen tot expressie gebracht in het cytoplasma vanaf 6 tot 12 uur na de inoculatie. Het eiwit verplaatste zich naar de kern van de cellen vanaf 12 tot 24 hpi. Vanaf 24 uur na inoculatie werd ook het replicatie-geassocieerde Rep eiwit gedetecteerd in de kern van de cellen. Het Rep eiwit werd tijdens de vroegste stadia van de infectie nooit opgemerkt in het cytoplasma van de geïnfecteerde cellen. Enkel in PK-15 cellen die afstierven ten gevolge van de infectie (te herkennen aan de gecondenseerde en gefragmenteerde kern) werd het Rep eiwit in lichte mate teruggevonden in het cytoplasma. Dit was waarschijnlijk het gevolg van lekkage vanuit de gedesorganiseerde kern. Een gelijkaardige opeenvolging van gebeurtenissen werd ook teruggevonden in de primaire cellijnen PAM en FCM. Bij deze cellen werden pas vanaf 48 uur na inoculatie virale eiwitten teruggevonden in de kern van de geïnfecteerde cel. In PAM werd bijkomend een belangrijke observatie gedaan. Er traden namelijk duidelijke verschillen op in gevoeligheid tegenover de PCV2-infectie tussen PAM afkomstig van verschillende biggen. In PAM van twee van de vijf geteste biggen konden nooit virale eiwitten in de kern van de geïnfecteerde cellen aangetoond worden, terwijl in PAM van de drie andere biggen tot 30% van de geïnfecteerde

cellen ook virale antigenen in de kern bevatten. Productie van nieuw virus kon in PK-15 cellen waargenomen worden vanaf 36 uur na inoculatie maar kon niet worden aangetoond in PAM of FCM culturen. In het tweede deel van de studie werd de vermeerderingskinetiek van zeven verschillende PCV2 stammen onderzocht in PK-15 cellen en onderling vergeleken. Er kon worden aangetoond dat twee abortus-geassocieerde stammen een afwijkende vermeerderingskinetiek vertoonden ten opzichte van de PMWS en PDNS-geassocieerde stammen. Na inoculatie van deze abortus-geassocieerde stammen werd een hoger aantal geïnfecteerde cellen geobserveerd op 24 uur na inoculatie. Het percentage van deze geïnfecteerde cellen dat virale eiwitten in de kern vertoonde, was echter kleiner in vergelijking met de andere stammen. Uit deze studie kon besloten worden dat de vermeerderingskinetiek van PCV2 in PAM en FCM gelijkaardig was maar trager verliep dan in de PK-15 cellen. Verder werd opgemerkt dat PAM afkomstig van verschillende varkens niet altijd even gevoelig waren voor infectie met PCV2. Dit kan erop wijzen dat de gevoeligheid van de macrofagen voor PCV2-infectie bepalend kan zijn voor de gevoeligheid van de gastheer zelf. Factoren die de status van de macrofaag zouden kunnen beïnvloeden, zouden op deze manier ook de gevoeligheid van het dier voor de PCV2-infectie kunnen beïnvloeden. De verschillen in vermeerderingskinetiek die waargenomen werden tussen de verschillende PCV2 stammen tonen aan dat ondanks de hoge genetische gelijkheid, mogelijk toch biologische verschillen tussen PCV2 stammen kunnen voorkomen.

Stimulatie van het immuunstelsel van het varken kan aanleiding geven tot een verhoogde vermeerdering van PCV2 in dat varken. De stimulatie van het immuunsysteem gaat altijd gepaard met de productie van een hele reeks van cytokines. Van een aantal van deze cytokines is geweten dat ze de vermeerdering van vele virale infecties kunnen tegengaan. Het lijkt dus contradictorisch dat PCV2 beter zou kunnen vermeerderen in een gastheer die deze antivirale cytokines produceert. Daarom werd een studie uitgevoerd die de invloed van een aantal cytokines op de vermeerdering van PCV2 naging. In hoofdstuk 4 wordt vooral de invloed van de verschillende interferonen toegelicht zowel *in vitro* als *in vivo*. In hoofdstuk 4.1 werd onderzocht of een reeks van recombinante porciene cytokines (interleukine-1 (IL-1), interleukine-6 (IL-6), interleukine-10 (IL-10), tumour necrosis factor-alpha (TNF-α),

interferon-alpha (IFN-α) en interferon-gamma (IFN-γ)) het verloop van een PCV2-infectie *in vitro* konden beïnvloeden. Hiervoor werden twee verschillende cellijnen gebruikt: de PK-15 cellijn en de 3D4/31 cellijn die van oorsprong een prociene monocytaire cellijn is. Wanneer celculturen behandeld werden met IL-1, IL-6, TNF-α of IL-10, werd geen invloed op het verloop van de PCV2-infectie vastgesteld. Daarentegen werd wel een effect vastgesteld wanneer de cellen behandeld werden met IFN-α of IFN-γ. Wanneer IFN-γ aan het medium van de cellen werd toegevoegd voor, tijdens of na de inoculatie, werd een verhoging van het aantal geïnfecteerde cellen opgemerkt tot respectievelijk 418, 171 en 691% in PK-15 en tot 706, 114 en 423% in 3D4/31 cellen. Een voorbehandeling van PK-15 cellen met IFN-α resulteerde in een daling van het aantal geïnfecteerde cellen. Maar wanneer het werd toegevoegd bij de cellen na de inoculatie steeg het aantal geïnfecteerde cellen tot 529% in PK-15 cellen en tot 308% in 3D4/31 cellen. Het effect van beide interferonen kon geneutraliseerd worden door hun respectievelijk neutraliserend monoklonaal antistof. Door middel van concanavalin A (ConA) stimulatie van perifere bloed mononucleaire cellen (PBMCs) werd supernatans verkregen dat natuurlijk porcien IFN-γ bevatte. Wanneer dit supernatans gebruikt werd om PK-15 cellen te behandelen dan werd een gelijkaardig effect bekomen als met het recombinante IFN-γ. Het effect geïnduceerd door het supernatans van de ConA-gestimuleerde PBMCs kon eveneens geneutraliseerd worden door IFN-γ neutraliserende monoklonale antistoffen. Behandeling van PK-15 cellen met IFN-γ leidde tot een 20 maal verhoogde productie van nieuw virus. Door middel van confocale microscopie en aan de hand van recombinant kapsied eiwitten die "virion like particles" (VLPs) vormen, kon vastgesteld worden dat het effect van IFN-γ op de PCV2-infectie vooral te wijten was aan een verhoogde internalisatie van de membraan-gebonden VLPs door de cellen. Er kon geen verandering vastgesteld worden ter hoogte van de binding van de VLPs aan de cel of ter hoogte van de productie van virale eiwitten na infectie met infectieus virus. Deze studie rapporteerde voor de eerste maal dat een behandeling met IFN-α en IFN-γ de vermeerdering van een virus verbetert.

In hoofdstuk 4.2 wordt een studie beschreven waarin gepoogd werd om de bevindingen die gedaan werden in hoofdstuk 4.1 te reproduceren *in vivo*. Gnotobiotische biggen werden intramusculair en subcutaan geïnoculeerd met ConA of recombinant IFN-γ (rIFN-γ) 12 uur voor de PCV2-inoculatie en gedurende 9 dagen

om de 3 dagen tijdens het verloop van de infectie. De vermeerdering van het virus werd gevolgd op drie verschillende tijdstippen door chirurgische biopsiename van de inguinale lymfeknopen op 10 en 15 dagen na de inoculatie (dpi) en bij de euthanasie op 21 dpi. In elk staal werd de hoeveelheid PCV2 bepaald door titratie van een 10% weefselsuspensie op PK-15 cellen. In de niet behandelde controlebiggen en in de biggen behandeld met rIFN-γ kon het virus pas worden teruggevonden in de inguinale lymfeknopen vanaf 15 dpi. Tussen deze groepen konden geen significante verschillen aangetoond worden. In biggen die behandeld werden met ConA kon het virus reeds vanaf 10 dpi teruggevonden worden. Op 15 dpi was de PCV2-vermeerdering hoger in deze biggen dan in de controlegroep. Ondanks de sterke verhoging van de virusvermeerdering onder invloed van ConA, konden in deze biggen geen klinische symptomen opgemerkt worden. Deze experimenten tonen aan dat een ConA-behandeling de vermeerdering van PCV2 *in vivo* kan stimuleren maar er kon niet uitgemaakt worden of deze stimulatie al dan niet afhankelijk was van de inductie van IFN-γ productie.

In hoofdstuk 5 werd de immuunrespons van het varken tegenover een PCV2-infectie onderzocht. Hoofdstuk 5.1 beschrijft de bevindingen die gedaan werden tijdens een studie waarin de cellulaire en humorale immuunrespons van individuele gnotobiotische biggen tegenover PCV2 werd onderzocht en gecorreleerd met de evolutie van de vermeerdering van het virus in het big. Twaalf gnotobiotische biggen werden geïnoculeerd met PCV2 waarvan 4 behandeld werden met cyclosporine A (CysA). CysA is een schimmeltoxine dat de stimulatie van T-cellen tegengaat en op die manier de cellulaire en thymus-afhankelijke humorale immuunrespons inhibeert. De CysA-behandelde dieren werden ingesloten om het effect van de specifieke immuniteit op het verloop van de PCV2-vermeerdering te onderzoeken. Door middel van inguinale lymfeknoop biopten kon de PCV2-vermeerdering opgevolgd worden op drie tijdstippen: 10, 15 en 21 dpi. De evolutie van de totale anti-PCV2 antistoffentiter (IPMA) en de neutraliserende antistoffentiter werd opgevolgd alsook de expressie van IFN-γ mRNA in PBMCs als een merker voor de cellulaire immuniteit. De CysA-behandelde biggen vertoonden de hoogste PCV2-vermeerdering, hetgeen aantoont dat de specifieke immuniteit zeer belangrijk is in het inperken van de virusvermeerdering. In de PCV2-geïnoculeerde dieren die niet behandeld werden met CysA, werden drie

verschillende vermeerderingspatronen opgemerkt. Patroon 1: PCV2 kon nooit geïsoleerd worden uit de lymfeknopen van 2 van de 8 biggen. Deze biggen werden wel geïnfecteerd met het virus vermits ze een duidelijke seroconversie vertoonden. Ze vertoonden zelfs de hoogste titers neutraliserende antistoffen tegenover PCV2 vanaf 15 dpi. Bij deze biggen werd een duidelijke piek in IFN-γ mRNA expressie aangetoond vanaf 15 dpi hetgeen een duidelijke indicatie geeft voor een goede cellulaire respons. Patroon 2: vijf biggen vertoonden lage tot matige PCV2-titers op 15 dpi die constant bleven of daalden naar 21 dpi toe. Deze biggen vertoonden duidelijke neutraliserende antistoffen maar de titers waren lager dan de titers bij de vorige biggen. Bij deze vijf dieren kon geen verhoging van de IFN-γ mRNA expressie aangetoond worden. Patroon 3: bij het laatste big werd een relatief lage PCV2 titer aangetroffen op 15 dpi. Deze titer vertoonde echter een sterke verhoging naar 21 dpi toe. Hoewel dit big duidelijk antistoffen tegenover PCV2 vertoonde (IPMA), kon er toch geen neutraliserende activiteit uitgaande van deze antistoffen aangetoond worden. Ook dit big vertoonde geen stijging in de expressie van IFN-γ mRNA. Deze studie toonde aan dat de aanvang van de specifieke immuunrespons tegenover PCV2 mogelijk verantwoordelijk is voor de variatie in het verloop van de vermeerdering van het virus in de gastheer. De afwezigheid van PCV2-neutraliserende antistoffen is mogelijk een zeer belangrijke factor in de pathogenese van de verhoogde PCV2-vermeerdering in een beperkt percentage van de biggen. Deze studie werd uitgevoerd in gnotobiotische biggen en er werd in het verleden reeds aangetoond dat de immuunrespons van dergelijke biggen erg kan afwijken van die van conventionele dieren. Daarom werd in hoofdstuk 5.2 onderzocht of gelijkaardige observaties konden gemaakt worden in conventionele dieren die natuurlijk of experimenteel geïnfecteerd werden met PCV2. In de studie die besproken wordt in dit hoofdstuk, werd aangetoond dat de correlatie tussen de afwezigheid van PCV2-neutraliserende antistoffen en het optreden van een verhoogde PCV2-vermeerdering in biggen ook terug te vinden was in conventionele dieren. In deze studie kon bijkomend een verband aangetoond worden tussen het afwezig zijn van de neutraliserende antistoffen en het optreden van PMWS in de biggen. De evolutie van de totale antistoffentiters verliep gelijkaardig bij subklinisch geïnfecteerde dieren en dieren die PMWS vertoonden. Beide groepen vertoonden seroconversie op hetzelfde tijdstip maar de titers bij de dieren met PMWS bleven iets lager. Bij de subklinisch geïnfecteerde

dieren liep de evolutie van de neutraliserende antistoffen samen met die van de totale antistoffen. Bij de dieren die PMWS vertoonden werd geen seroconversie in neutraliserende antistoffen aangetroffen of konden ze enkel gedurende een korte tijd gedetecteerd worden. Wanneer de verschillende antistof isotypes onderzocht werden, kwamen duidelijke verschillen naar voren. Bij subklinisch geïnfecteerde dieren bleven de IgM antistoffen tegenover PCV2 aanwezig tot aan het einde van elke studie. Bij de dieren met PMWS werden ook IgM antistoffen gedetecteerd maar deze verdwenen snel of bleven aanwezig in zeer lage titers. Verder werd ook duidelijk dat de gemiddelde titers van de andere onderzochte antistof isotypes lager lagen bij de dieren die PMWS vertoonden dan bij de subklinisch geïnfecteerde dieren. Deze verschillen waren echter maar in enkele gevallen significant.

De algemene conclusie van deze thesis is dat de vermeerdering van PCV2 in zijn gastheer sterk beïnvloed wordt door verscheidene gastheer-specifieke en externe factoren. Er werd aangetoond dat in verschillende experimentele en veldstudies een deel van de biggen niet in staat was om een efficiënte immuunrespons tegenover PCV2 op te starten vermits ze geen neutraliserende antistoffen konden aanmaken. Dit resulteerde bij deze biggen in een sterk verhoogde vermeerdering van PCV2 en leidde in enkele studies tot het optreden van PMWS. Dit verschil in capaciteit om PCV2-neutraliserende antistoffen aan te maken kan mogelijk de basis leggen voor het identificeren van de genetische invloed op de gevoeligheid van varkens voor PMWS. Verder werd in dit proefschrift ook aangetoond dat IFN-γ en in meer specifieke condities ook IFN-α de gevoeligheid van twee continue varkenscellijnen tegenover PCV2 konden verhogen. Er werden ook indicaties gevonden dat een gelijkaardig mechanisme *in vivo* een rol zou kunnen spelen. Toekomstig werk gebaseerd op de bevindingen die gemaakt werden tijdens dit werk, zou kunnen leiden tot de kennis om strategieën uit te werken om een varkenslijn te selecteren die resistent zou zijn aan een verhoogde PCV2-vermeerdering en dus aan PMWS. Verder kunnen op basis van deze bevindingen ook sanitaire maatregelen aanbevolen worden die kunnen leiden tot een lagere prevalentie van PMWS in de huidige varkensstapel.

Curriculum Vitae

PERSONALIA

Peter Meerts werd op 27 december 1976 geboren te Ukkel. In 1994 beëindigde hij zijn secundaire opleiding aan het Sint-Victor Instituut te Alsemberg in de richting Latijn-Wiskunde-Wetenschappen. In 2001 behaalde hij het diploma van dierenarts aan de Faculteit Diergeneeskunde van de Universiteit Gent. Vanaf augustus 2001 tot juli 2005 was hij tewerkgesteld in het Laboratorium voor Virologie als wetenschappelijk medewerker op het project "Infectieus groeistopsyndroom, een circovirus-aandoening bij biggen: pathogenese, diagnose en bestrijding" van de Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu. In het kader van dit project deed hij vooral onderzoek naar de interactie tussen het porcien circovirus type 2 en de immuunrespons van het varken. Dit werk leidde tot auteur of co-auteurschap van 10 publicaties in internationale wetenschappelijke tijdschriften. Hij nam deel aan 2 internationale wetenschappelijke congressen en gaf verscheidene voordrachten over virale ziekten bij het varken aan dierenartsen in verschillende landen. Een deel van het werk dat hij uitvoerde in verband met de invloed van interferon-gamma op de circovirusinfectie, gaf aanleiding tot het indienen van het patent getiteld "Culturing circular ssDNA viruses for the production of vaccines".

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PATENT

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Dankwoord

Het einde van een doctoraat is het ideale moment om terug te kijken op een belangrijke periode. Hoewel deze periode vooral extra vragen heeft voortgebracht is ze toch zeer verrijkend geweest zowel op professioneel als op persoonlijk vlak. Het is de periode geweest waarin ik mocht, kon of moest samenwerken met mensen van verschillende pluimage en dat maakte het werk enorm boeiend. Dit is dan ook het ideale moment om deze mensen te danken voor hun aandeel in dit werk.

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A PhD work can only reach an acceptable level when its observations are being discussed with external experts. I was proud to find Professor Segalés willing to judge my work. I have always admired his critical way of dealing with the immensely

complex matter of PMWS and I acknowledge him as one of the most important researchers in the PCV2-PMWS community. Gelukkig kon ik ook dichterbij huis beroep doen op de hulp van experts. Zo speelde een groot deel van mijn werk zich af in de immunologie en dan kon ik steeds rekenen op Professor Cox. Onze samenwerking is steeds zeer vlot verlopen, zodanig zelfs dat ik niet enkel zijn PCR maar ook zijn laborante ter beschikking kreeg. Voor deze samenwerking wil ik zowel Prof. Cox als Griet bedanken.

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ben. Natuurlijk dank ik het ministerie van landbouw (later het ministerie van volksgezondheid) om mij de kans te geven om gedurende al die jaren dit onderzoek uit te voeren.

The research that I was able to perform and the results that came out of it are certainly not the result of my project alone. The observations that led to the golden hypothesis, were made by Dr. Romeo Sanchez. Romeo, together we went through a few tough years of PCV2 research. I'm sure you still remember them. I have always appreciated the fact that, although you were more than busy with getting your own publications, you still had time to give me the proper training I needed to get started myself. For this I cannot thank you enough. Know that this thesis would not have existed if you wouldn't have done the basic research before.

I am reassured to know that the PCV2 work will not be abandoned. In fact it will be continued by two guys with whome I had a lot of fun the last few years. Misinzo, although you look a bit darker compared to the rest of us, you really lightened up the lab. Countless times you were the real centre of the lab with jour stories about Africa. Whenever I get caught by a Boa Constrictor, I'll be able to survive only thanks to your good advice. You had the amazing ability to blend into the lab the minute you arrived. I still remember the first dutch words you learned... and I will never forget the meaning of mbusi katoloki. Besides being a very good collegue, you are also a good scientist. I admired the way you were able to produce such high quality results once you had set your mind to it. David is degene die echt de fakkel overneemt. In het begin vroeg ik mij sterk af of de serieuze wetenschap wel te combineren viel met zo'n bedenkelijk gevoel voor humor. Blijkbaar wel. Tijdens de periode dat ik geen tijd meer had om nog veel in het labo rond te lopen, zag ik wel dat je je weg snel zelf zou vinden. Ook zag ik dat je snel charmanter gezelschap opzocht om mee te "werken". Ik wens je veel succes, geluk (en sterkte) in het verdere PCV2-onderzoek.

In mijn wetenschappelijke hindernissenkoers kon ik steeds rekenen op de hulp van drie van mijn mede-doctoraatsstudenten. Wanneer ik eraan terugdenk dan moet Steven wel dikwijls gedacht hebben: "Is hij daar nu wéér met zijn gezaag over dat onnozel virus?". De gesprekken over onzer beider problemen op het labo hebben mij dikwijls geholpen om oplossingen te vinden, of toch tenminste om de moed terug te vinden om ernaar te zoeken. Mensen zoals jij waren onontbeerlijk om mijn doctoraat af te werken. Mijn andere trouwe toeverlaat was steeds Putti alias Peter D. Telkens als ik mij durfde (of moest) in het moleculaire doolhof wagen, was hij er het eerste

slachtoffer van. Ik verbaasde mij steeds weer over het enthousiasme en geduld waarmee hij mij de finesse van de moleculaire technieken trachtte bij te brengen. En dat terwijl een dierenarts daar toch niet echt voor gemaakt is… Ik troost mij met de gedachte dat het bloed trekken bij u net zo goed ging als het western blotten bij mij. Het ging wel maar ik was toch niet graag dat varken geweest… Aan wie ik natuurlijk niets meer moest vertellen in verband met de praktische kant van het onderzoek, was Geoffrey. Hoewel hij de bescheidenheid zelve was, moet hij één van de meeste precieze onderzoekers geweest zijn die op het labo rondliepen. Verder was zijn specialiteit het opsporen van typfouten en spellingsfouten waarvan we nadien deden alsof het typfouten waren. Geoffrey, ik hoop dat je mijn thesis niet te nauwkeurig naleest.

Diergeneeskundig onderzoek kan natuurlijk niet zonder een hoop praktisch werk. Hiervoor kon ik steeds rekenen op de hulp van twee zeer geroutineerde en ronduit fantastische dierenverzorgers. Hoewel Fernand zijn specialiteit vooral lag in het zachtaardig omgaan met katten, had hij toch ook een zeer brede kennis over de sociale omgang met varkens. Hij was dan ook de geknipte (niet letterlijk te nemen) persoon om mij, na mijn theoretische studies, de praktische zaken bij te leren. Zoals zo vaak bleek de theorie weer niet op te wegen tegen jarenlange ervaring. Geert Surété had ook zo'n ervaring in de omgang met dieren, maar dan vooral met de dode versie ervan. Het was altijd een hilarische ervaring om samen met hem den boer op te gaan. Samen hebben we er ook altijd voor gezorgd dat de mensen die niet in contact kwamen met varkens, regelmatig met de neus op de feiten werden gedrukt. Fernand en Geert vormden samen de "Laurel en Hardy" (zelf in te vullen wie wie is) van de virologie. Als het urenlange schijven mij teveel werd, kon ik altijd eens wat tipkes gaan vullen en voor ik het wist waren we een uur en verscheidene breuken van het lachen verder.

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keer een team hé zeg". PRRSV en macrofagen waren de specialiteit van Chantal; bioassay's, cytokines en augustijn die van Lieve. Met z'n allen vormden ze een team waarop iedereen steeds beroep kon doen. Het was dan ook met gemengde gevoelens dat ik dat team achterliet omdat ik besef dat zo'n samenwerking niet evident is. Ook de nieuwste kracht, Nele, heeft al aangetoond dat ze een waardevolle aanwinst is.

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I owe special thanks to my Danish collegues and not only for the nice week I spent at Lindholm. The work that they performed on PCV2 was another key to come to the golden hypothesis. I believe this is well illustrated in this thesis since I have referred 24 times to the publication of Ann-Sofie.

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De wetenschap mag weten,
het wonder blijft bestaan.
Ik weet maar één ding zeker,
de rest mag je vergeten.
Eén waarheid blijft bestaan:
we komen en we gaan.

***Stef Bos**, Vuur*